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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/00, 15/12, A61K 39/00	A1	(11) International Publication Number: **WO 99/23214**
		(43) International Publication Date: 14 May 1999 (14.05.99)

(21) International Application Number: PCT/US98/23170

(22) International Filing Date: 30 October 1998 (30.10.98)

(30) Priority Data:
60/064,329 30 October 1997 (30.10.97) US

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: COMPOSITIONS AND METHODS FOR ENHANCING CARDIAC CONTRACTILITY MEDIATED BY MYOCYTE P2 PURINOCEPTORS AND MODELS THEREOF

(57) Abstract

A recombinant myocyte comprising at least one introduced nucleic acid encoding a P2 purinoceptor, such as a $P2X_4$ or $P2X_6$ purinoceptor, and a method of determining whether a compound affects cardiac contractility in an animal are provided. Furthermore, a method of augmenting cardiac contractility and a method of treating heart failure are included in the invention, as is a kit comprising one or more recombinant myocytes and an instructional material.

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COMPOSITIONS AND METHODS FOR ENHANCING CARDIAC CONTRACTILITY MEDIATED BY MYOCYTE P2 PURINOCEPTORS AND MODELS THEREOF

FIELD OF THE INVENTION

The field of the invention is cardiac myocyte contractility.

BACKGROUND OF THE INVENTION

Heart failure is the cause of significant morbidity and mortality in humans, other mammals, and other animals. Heart failure is a clinical syndrome having many different etiologies. This syndrome arises from abnormality(ies) in the mechanical performance of the heart, which reduces cardiac output to a level that is insufficient to meet the body's needs for oxygen supply and waste (e.g. CO_2) removal. Impaired cardiac function may be manifested only during exertion or, particularly in severe cases of heart failure, even when the animal is at rest. Heart failure frequently arises in conjunction with valvular, coronary, or myocardial diseases, and may furthermore be caused or aggravated by arrhythmias.

With limited exceptions, heart failure is associated with a deficiency in the contractility of cardiac myocytes, particularly in the contractility of myocytes of the mammalian (including human) left ventricle. Loss of contractility of myocytes of the mammalian right ventricle may also result in heart failure, and often arises as a consequence of persistent left ventricle failure. Clinical symptoms of heart failure are well known, and include undue tachycardia, fatigue on exertion, dyspnea on mild exercise, and intolerance to cold. Humans experiencing heart failure sometimes also exhibit pulmonary edema and associated symptoms.

Known treatments for heart failure include rest, oxygenation, correction of arrhythmias, measures to improve myocardial contractility, diuresis, and reduction of circulatory preload and afterload. Digitalis preparations, such as digoxin, digitoxin,

and quabain, are the most common agents used to enhance myocardial contractility. However, because therapeutic doses of digitalis preparations are very near toxic doses, digitalis toxicity is a common drawback to such treatment and may preclude its use in certain patients. What is needed are agents which may be used to enhance cardiac contractility without causing the toxicity associated with digitalis preparations. The present invention satisfies this need by providing such agents, kits, methods, and model systems for identifying such agents.

Cardiac Purinoceptors

Adenosine 5'-triphosphate (ATP) exerts a number of pronounced effects in the cardiovascular system (Olsson et al., 1990, Physiol. Rev. 70:761-846; Ralevic et al., 1991, Circ. 84:1-14). These effects include stimulatory effects in the heart such as vasodilatation in the coronary vasculature, stimulation of transsarcolemmal calcium entry into cardiac myocytes, acidification and depolarization of cardiac cells, cytosolic calcium transients, and a pronounced positive inotropic effect in the cardiac myocyte (Olsson et al., 1990, Physiol. Rev. 70:761-846; Ralevic et al., 1991, Circ. 84:1-14; Scamps et al., 1990, Circ. Res 67:1007-1016; Scamps et al., 1994, Br. J. Pharmacol. 113:982-986; Scamps et al., 1992, J. Gen. Physiol. 100:675-701; Puceat et al., 1991, Biochem. J. 274:55-62; Scamps et al., 1990, Pflugers Arch. 417:309-316; Danziger et al., 1988, Cell Calcium 9:193-199; DeYoung et al., 1989, Am. J. Physiol. 257(Cell Physiol. 26):C750-C758; Forrester et al., 1977, J. Physiol. (London) 268:371-390). This latter effect is mediated by a class of cell surface ATP receptors designated P2 purinoceptors (Danziger et al., 1988, Cell Calcium 9:193-199; Leggssyer et al., 1988, J. Physiol. 401:185-199).

Physiologically, ATP is released from platelets, endothelial cells, or hypoxic cardiac tissues and acts as a paracrine and autocrine regulatory agent (Borst et al., 1991, Circ. Res. 68:797-806; Clemens et al., 1980, J. Physiol. (London) 312:143-158; Forrester et al., 1977, J. Physiol. (London) 268:371-390; Fredholm et al., 1982, Acta Physiol. Scand. 116:285-295; Pearson et al., 1979, Nature 281:384-386; Vial et al., 1987, J. Mol. Cell. Cardiol. 19:187-197). Binding of ATP to P2

purinoceptors provides important inotropic support in both healthy and diseased heart tissue. When released by norepinephrine from sympathetic nerve endings, ATP is capable of acting synergistically with one or more β-adrenergic agonists to augment myocyte contractility (Zheng et al., 1992, Am. J. Physiol. 262(Cell Physiol. 31):C128-C135). The mechanism by which ATP increases myocyte contractility is not well understood.

A number of cDNA molecules have been cloned which encode P2 purinoceptors. cDNA molecules encoding at least four P2Y receptor subtypes, seven P2X receptor subtypes, and the P2 receptor found on macrophages and platelets have been cloned (Burnstock et al., 1996, Drug Develop. Res., 38:67-71).

Activation of the P2Y receptor has been shown to variably inhibit adenylyl cyclase (AC) activity and cyclic adenosine monophosphate (cAMP) accumulation, or to have no effect on AC activity and cAMP accumulation (Scamps et al., 1992, J. Gen. Physiol. 100:675-701; Yamada et al., 1992, Circ. Res. 70:477-485). P2Y receptor activation is coupled to inhibition of AC activity and cAMP accumulation via inhibitory G protein (Gi).

P2Y receptor activation is also linked to stimulation of phosphatidyl inositol 4,5-bisphosphate phospholipase C (PIP2-PLC) activity. The role of PIP2-PLC in mediating P2 receptor agonist-stimulated myocyte contractility has not been clearly understood by others. Physiologically, stimulation of PIP2-PLC activity results in intracellular accumulation of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG; Harden et al., 1995, Annu. Rev. Pharmacol. Toxicol. 35:541-579). It is possible that $InsP_3$ stimulates myocyte contractility by mobilizing intracellular calcium and that DAG increases myofilament sensitivity to calcium by activating protein kinase C (PKC), thereby enhancing cardiac contractility.

P2X receptors are a family of related ligand-gated ion channel proteins which facilitate entry of sodium and calcium ions into the cell (Brake et al., 1994, Nature 371:519-523; Chen et al., 1995, Nature 377:428-431; Valera et al., Nature

371:516-519). P2X receptor proteins are known to be present physiologically in the form of homodimers and heterodimers (e.g. a $P2X_2/P2X_3$ heterodimer).

Although it appears that P2 purinoceptors mediate stimulation of calcium entry into and accumulation within the cytosolic compartment of isolated cardiac myocytes, the subtype of P2 purinoceptor that mediates increases in cardiac myocyte contractility and the mechanism underlying this stimulatory effect have, until the present disclosure, remained unknown. Reported studies investigated the effects of ATP and ATP analogs on contractility of rat cardiac ventricular myocytes and intact papillary muscle (Danziger et al., 1988, Cell Calcium 9:193-199; Leggssyer et al., 1988, J. Physiol. 401:185-199; Scamps et al., 1990, Circ. Res 67:1007-1016). These studies clearly demonstrate that a positive inotropic response is induced by ATP. Although it was suggested that a P2Y receptor mediated the increase in the cytosolic calcium level (Bjornsson et al., 1989, Eur. J. Biochem. 186:395-404), the nature of the P2 receptor has not been determined. The cellular mechanism underlying the positive inotropic effect of ATP on cardiac myocytes remains poorly understood by others. This lack of understanding is due, at least in part, to the absence of a myocyte model for the cardiac P2 purinoceptor. Such a model would be useful for identifying compounds which affect the function of the P2 purinoceptor. The present invention provides such a model.

SUMMARY OF THE INVENTION

The invention relates to a recombinant myocyte comprising at least one introduced nucleic acid which encodes at least one P2 purinoceptor. The purinoceptor may, for example, be selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, a $P2X_4$ purinoceptor, a $P2X_5$ purinoceptor, and a $P2X_6$ purinoceptor. Preferably, the P2 purinoceptor is a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. The myocyte may, for example, be a chicken embryonic ventricular myocyte or a rat ventricular myocyte.

The invention also relates to a method of determining whether a compound enhances cardiac contractility. This method comprises assessing the contractile amplitude of a recombinant myocyte in the presence and absence of the compound. The myocyte comprises at least one first introduced nucleic acid encoding at least one of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor. An increase in the contractile amplitude of the myocyte in the presence of the compound, relative to the contractile amplitude of the myocyte in the absence of the compound, is an indication that the compound enhances cardiac contractility. Preferably, the myocyte is a chicken embryonic ventricular myocyte or a rat ventricular myocyte.

In an alternate embodiment, this method further comprises assessing the contractile amplitude of a non-recombinant myocyte in the presence and absence of the compound. If the difference between the contractile amplitude of the recombinant myocyte in the presence of the compound and the contractile amplitude of the recombinant myocyte in the absence of the compound is greater than the difference between the contractile amplitude of the non-recombinant myocyte in the presence of the compound and the contractile amplitude of the non-recombinant myocyte in the absence of the compound, then the effect of the composition on contractile amplitude is attributable to the at least one purinoceptor.

The invention further relates to a method of determining whether a compound enhances cardiac contractility. This method comprises assessing calcium uptake by a recombinant myocyte in the presence and absence of the compound. The myocyte comprises at least one first introduced nucleic acid encoding at least one of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor. An increase in calcium uptake by the myocyte in the presence of the compound, relative to calcium uptake by the myocyte in the absence of the compound, is an indication that the compound enhances cardiac contractility. In an alternate embodiment of this method calcium uptake by a non-recombinant myocyte in the presence and absence of the compound is also assessed. If the difference between calcium uptake by the recombinant myocyte in the presence of the compound and calcium uptake by the recombinant myocyte in the absence of the

compound is greater than the difference between calcium uptake by the non-recombinant myocyte in the presence of the compound and calcium uptake by the non-recombinant myocyte in the absence of the compound, then the effect of the composition on calcium uptake is attributable to the at least one purinoceptor.

The invention still further relates to a method of determining whether a compound enhances cardiac contractility. This method comprises assessing the contractile amplitude of a first myocyte and a second myocyte in the presence and absence of the compound. The first myocyte comprises at least one first P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, and a $P2X_4/P2X_6$ heterodimer. The second myocyte comprises the at least one first P2 purinoceptor and at least one introduced nucleic acid encoding at least one second P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. If the difference between the contractile amplitude of the second myocyte in the presence of the compound and the contractile amplitude of the second myocyte in the absence of the compound is greater than the difference between the contractile amplitude of the first myocyte in the presence of the compound and the contractile amplitude of the first myocyte in the absence of the compound, then the compound enhances cardiac contractility. The second P2 purinoceptor is preferably selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, and a $P2X_5$ purinoceptor.

The invention yet further relates to a method of determining whether a compound enhances cardiac contractility. This method comprises assessing calcium uptake by a first myocyte and a second myocyte in the presence and absence of the compound. The first myocyte comprises at least one first P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, and a $P2X_4/P2X_6$ heterodimer. The second myocyte comprises the at least one first P2 purinoceptor and at least one introduced nucleic acid encoding at least one second P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. If the difference between calcium uptake by the second myocyte in the presence of the compound and

calcium uptake by the second myocyte in the absence of the compound is greater than the difference between calcium uptake by the first myocyte in the presence of the compound and calcium uptake by the first myocyte in the absence of the compound, then the compound enhances cardiac contractility. In this method, the second P2 purinoceptor is preferably selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, and a $P2X_5$ purinoceptor.

The invention also relates to a method of enhancing cardiac contractility in an animal. This method comprises administering to the animal a composition comprising at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, and an antagonist of a P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. Following administration of the compound, cardiac contractility is augmented in the animal. The at least one agent is preferably selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist, for example, 2-methylthio-ATP (2-MeSATP). When 2-MeSATP is administered to the animal, it is preferably administered in an amount sufficient to effect a concentration of at least about 0.3 micromolar, and preferably at least about 3 micromolar, 2-MeSATP in a cardiac tissue of the animal. In an alternate embodiment of this method, at least one antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor is administered to the animal. This antagonist is preferably an antagonist of a P2 purinoceptor selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, and a $P2X_5$ purinoceptor, for example, suramin, reactive blue-2, or pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid. Also preferably, the at least one antagonist is administered in an amount sufficient to effect a concentration of at least about 30 micromolar, and more preferably 300 micromolar antagonist in a cardiac tissue of the animal. Preferably, the animal is a mammal, such as a human.

The invention further relates to a pharmaceutical composition for enhancing cardiac contractility. This pharmaceutical composition comprises at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, and an antagonist of a P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. Preferably, at least one agent is selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist. Also preferably, the composition comprises both a $P2X_4$ or $P2X_6$ purinoceptor agonist and at least one antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

The invention still further relates to a kit comprising a first container containing at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist and a second container containing at least one antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

The invention yet further relates to a method of treating an animal experiencing heart failure. This method comprises administering to the animal a composition comprising at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, and an antagonist of a P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. Preferably, at least one agent is selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist. Also preferably, both a $P2X_4$ or $P2X_6$ purinoceptor agonist and at least one antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor are administered to the animal.

The invention also relates to a kit for determining whether a compound enhances cardiac contractility. This kit comprises a recombinant myocyte comprising at least one introduced nucleic acid encoding at least one P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor. The kit also comprises an instructional material which describes assessment of at least one of the contractile amplitude of the myocyte and calcium uptake by the myocyte.

The invention further relates to a kit for determining whether a compound enhances cardiac contractility. This kit comprises a first myocyte and a second myocyte. The first myocyte comprises at least one first P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, and a $P2X_4/P2X_6$ heterodimer. The second myocyte comprises at least one introduced nucleic acid encoding at least one second P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

The invention still further relates to a method of determining whether a compound decreases cardiac contractility. This method comprises assessing the contractile amplitude of a recombinant myocyte in the presence and absence of the compound. The myocyte comprises at least one first introduced nucleic acid encoding at least one of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor. A decrease in the contractile amplitude of the myocyte in the presence of the compound, relative to the contractile amplitude of the myocyte in the absence of the compound, is an indication that the compound decreases cardiac contractility. In an alternate embodiment of this method, the contractile amplitude of a non-recombinant myocyte is assessed in the presence and absence of the compound. If the decrease in the contractile amplitude of the recombinant myocyte in the presence of the compound, relative to the decrease in the contractile amplitude of the recombinant myocyte in the absence of the compound, is greater than the decrease in the contractile amplitude of the non-recombinant myocyte in the presence of the compound, relative to the decrease in the contractile amplitude of the non-recombinant myocyte in the absence of the compound, then the effect of the composition on contractile amplitude is attributable to the at least one purinoceptor.

The invention also relates to a method of decreasing cardiac contractility in an animal. This method comprises administering to the animal a composition comprising at least one agent selected from the group consisting of a $P2X_4$ purinoceptor antagonist, a $P2X_6$ purinoceptor antagonist, and an agonist of a P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. Following

administration of the composition to the animal, cardiac contractility is deereased in the animal.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1, comprising Figures 1A and 1B, depicts the effects of adenine nucleotides on cultured cardiac ventricle myocyte contractile amplitude. Figure 1A is a graph which illustrates the effects of ATP, ADP, and AMP on the basal level of cardiac ventricular myocyte contractility. Figure 1B is a tracing which depicts cardiac myocyte contractility before and after treating a myocyte with 1 micromolar ATP. As illustrated in Figure 1B, the contractile amplitude was approximately 1 micrometer prior to treatment with ATP and approximately 1.8 micrometers following treatment with ATP, representing an approximately 80% increase in contractile amplitude.

Figure 2 is a graph which depicts the effects of P2 purinoceptor-selective agonists on contractile amplitude in cultured cardiac ventricular myocytes.

Figure 3, comprising Figures 3A and 3B, depicts the effects of ATP, UTP, and 2-MeSATP on intracellular inositol phosphate levels in cultured cardiac ventricular myocytes. In the graph depicted in Figure 3A, myocytes were exposed to 100 micromolar ATP (filled squares) for 30 seconds or maintained in medium not containing ATP (open squares), and the intracellular levels of $InsP_1$, InsP2, and $InsP_3$ were assessed. In the graph depicted in Figure 3B, the effects of exposing myocytes to varying concentrations of ATP, UTP, or 2-MeSATP on the intracellular level of inositol phosphates (i.e. including $InsP_1$, InsP2, and $InsP_3$) are illustrated.

Figure 4 is a bar graph which illustrates cross-desensitization of phosphoinositide hydrolysis induced by UTP and 2-MeSATP, as described herein.

Figure 5, comprising Figures 5A, 5B, 5C, and 5D, is a series of tracings which depict the effect of pretreatment with 2-MeSATP and UTP on the subsequent inotropic effect caused by addition of 2-MeSATP or UTP. The tracings depicted in Figure 5A indicate the contractile amplitude of cultured ventricular myocytes which

were pre-incubated with 100 micromolar 2-MeSATP for 18 minutes and subsequently re-challenged with 1 micromolar 2-MeSATP. The tracings depicted in Figure 5B indicate the contractile amplitude of cultured ventricular myocytes which were pre-incubated with 100 micromolar 2-MeSATP for 18 minutes and subsequently re-challenged with 10 micromolar UTP. The tracings depicted in Figure 5C indicate the contractile amplitude of cultured ventricular myocytes which were pre-incubated with 100 micromolar UTP for 19 minutes and subsequently re-challenged with 1 micromolar 2-MeSATP. The tracings depicted in Figure 5D indicate the contractile amplitude of cultured ventricular myocytes which were pre-incubated with 100 micromolar UTP for 19 minutes and subsequently re-challenged with 10 micromolar UTP.

Figure 6 is a series of tracings which depict the effect of U-73122 on the 2-MeSATP-stimulated increase in contractile amplitude of cultured cardiac ventricular myocytes.

Figure 7, comprising Figures 7A and 7B, is a pair of graphs which depict the effects of P2 purinoceptor agonists on ^{45}Ca uptake by cardiac ventricular cells. The graph depicted in Figure 7A illustrates ^{45}Ca uptake by cardiac myocytes treated with 1 micromolar 2-MeSATP (filled circles), relative to ^{45}Ca uptake by cardiac myocytes maintained in medium which did not contain 2-MeSATP (open circles). The graph depicted in Figure 7B illustrates the effects of 1 micromolar α,β-methylene-ATP on ^{45}Ca uptake by cardiac myocytes exposed to the compound (filled circles) or maintained in medium not containing the compound (open circles).

Figure 8, comprising Figures 8A and 8B is a pair of tracings which depict the effects of ATP and 2-MeSATP on the contractile amplitude of rat cardiac ventricular myocytes. The contractile amplitude of myocytes before and after addition of 0.3 micromolar ATP is illustrated in Figure 8A. The contractile amplitude of myocytes before and after addition of 1 micromolar 2-MeSATP is illustrated in Figure 8B.

Figure 9, comprising Figures 9A, 9B, and 9C is a trio of tracings which depict the effects of the P2 receptor antagonists, on the contractile amplitude of rat cardiac ventricular myocytes. The contractile amplitudes of myocytes exposed to 1 micromolar 2-MeSATP and then to 1 micromolar 2-MeSATP and 100 micromolar PPADS are illustrated in Figure 9A. The contractile amplitude of myocytes exposed to 1 micromolar 2-MeSATP and then to 1 micromolar 2-MeSATP and 100 micromolar suramin are illustrated in Figure 9B. The contractile amplitude of myocytes exposed to 10 micromolar U-73122 and then to 10 micromolar U-73122 and 1 micromolar 2-MeSATP are illustrated in Figure 9C.

Figure 10 is a graph which depicts the effect of 2-MeSATP on calcium influx of transfected cardiac ventricular myocytes. Data obtained using myocytes transfected with a vector encoding $P2X_4$ receptor are indicated by filled circles. Data obtained using myocytes transfected with a vector which did not encode $P2X_4$ receptor are indicated by filled squares.

Figure 11, comprising Figures 11A, 11B, 11C, 11D, 11E, and 11F, is a series of tracings depicting the effect of 2-MeSATP on the contractile amplitude of transfected cardiac ventricular myocytes. Data obtained using myocytes transfected with a vector encoding $P2X_4$ receptor are presented in Figures 11D, 11E, and 11F. Data obtained using myocytes transfected with a vector which did not encode $P2X_4$ receptor are presented in Figures 11A, 11B, and 11C.

DETAILED DESCRIPTION

The invention relates to the discovery that cardiac myocontractility in animals such as mammals, and particularly in humans, can be enhanced by administering to cardiac myocytes an agonist of one or both of the $P2X_4$ and $P2X_6$ purinoceptor subtypes, and that cardiac myocontractility in such animals can alternately, or further, be enhanced by administering an antagonist of a P2 purinoceptor other than the $P2X_4$ and $P2X_6$ purinoceptor subtypes to the cardiac muscle cells. Thus, known $P2X_4$ and $P2X_6$ agonist compounds, such as 2-methylthio-ATP, can be

used in place of highly toxic digitalis compositions to treat cardiac failure. Heart failure can furthermore be treated by administering to a patient experiencing, for example, arrhythmia-induced congestive heart failure a composition comprising a $P2X_4$ or $P2X_6$ agonist agent and one or more known antagonists of another P2X receptor, such as suramin, reactive blue-2, or PPADS. Alternately, one or more of the antagonists may be administered without administering an agonist.

Because only a limited number of P2 receptor agonists and antagonists are presently known, the invention further includes a recombinant myocyte model and a screening method of using such recombinant myocytes to identify compounds which enhance myocyte contractility. It will be understood by the skilled artisan that such methods can be used without adaptation to identify compounds which diminish cardiac myocyte contractility, if such compounds are desired. Such compounds include $P2X_4$ antagonists and $P2X_6$ antagonists, and are useful, for example, to treat patients afflicted with ischemic heart disease.

This screening method comprises assessing the contractile amplitude of a recombinant myocyte of the invention comprising an introduced nucleic acid encoding at least one of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor. The contractile amplitude of this recombinant myocyte is assessed in the presence and absence of a test compound. If the contractile amplitude of the recombinant myocyte in the presence of the test compound is greater than the contractile amplitude of the recombinant myocyte in the absence of the test compound, then this is an indication that the test compound enhances cardiac contractility. If the contractile amplitude of the recombinant myocyte in the presence of the test compound is less than the contractile amplitude of the recombinant myocyte in the absence of the test compound, then this is an indication that the test compound diminishes or inhibits cardiac contractility. When the myocyte is cultured on a surface, the contractile amplitude of the recombinant myocyte is preferably assessed using an opticovideo motion-detection system, as described (Barry et al., 1982, J. Physiol. 325:243-260). However, it is understood that any method of assessing muscle cell contractility may be used.

Alternately, according to this screening method, the change in the contractile amplitude of the recombinant myocyte attributable to the presence of the test compound may be compared with the change in the contractile amplitude of a non-recombinant myocyte (i.e. a myocyte which does not comprise the introduced nucleic acid) attributable to the presence of the test compound. If the contractile amplitude of the recombinant myocyte in the presence of the test compound is greater than the contractile amplitude of the non-recombinant myocyte in the presence of the test compound, then this is an indication that the test compound enhances cardiac contractility. If the difference between the contractile amplitude of the recombinant myocyte in the presence of the test compound and the contractile amplitude of the recombinant myocyte in the absence of the test compound, is greater than the difference between the contractile amplitude of the non-recombinant myocyte in the presence of the test compound and the contractile amplitude of the non-recombinant myocyte in the absence of the test compound, then this is also an indication that the test compound enhances cardiac contractility.

The contractile amplitude of the myocyte may be assessed using any method known in the art including, but not limited, the methods described herein. Thus, the contractile amplitude may be assessed by microscopically examining the myocyte, by using an opticovideo motion-detection system, and the like.

Calcium uptake by myocytes may be assessed instead of contractile amplitude, it being understood that increased calcium uptake is associated with increased contractility in myocytes such as cardiac myocytes. Therefore, assessment of calcium uptake may be performed in place of assessment of contractile amplitude wherever assessment of contractile amplitude is mentioned in the present disclosure.

In these assay methods, the myocyte may comprise an introduced nucleic acid encoding one purinoceptor, an introduced nucleic acid encoding a plurality of purinoceptors, or a plurality of introduced nucleic acids, each encoding the same or different purinoceptors, as described elsewhere herein.

When myocytes comprising an introduced nucleic acid encoding one purinoceptor are used, it is possible to assess the effect of test compounds on contractile amplitude modifications attributable to that receptor. Thus, for example, using a first recombinant myocyte comprising an introduced nucleic acid encoding a $P2X_4$ purinoceptor and a second recombinant myocyte comprising an introduced nucleic acid encoding both a $P2X_4$ purinoceptor and a $P2X_5$ purinoceptor, it is possible to identify compounds which are not $P2X_4$ agonists, but which, in the presence of a $P2X_4$ agonist, further enhance cardiac myocyte contractility by virtue of being $P2X_5$ antagonists.

The screening method may be performed using a first myocyte comprising a $P2X_4$ or $P2X_6$ purinoceptor and a second (recombinant) myocyte comprising the same $P2X_4$ or $P2X_6$ purinoceptor and an introduced nucleic acid encoding one or more P2 purinoceptors other than the $P2X_4$ or $P2X_6$ purinoceptor. The first myocyte may be recombinant or not. The screening method is performed by assessing contractility of the first myocyte in the presence of an agonist of the $P2X_4$ or $P2X_6$ purinoceptor and in the presence and absence of a test compound and contractility of the second myocyte in the presence of the agonist and in the presence and absence of the test compound. If contractility is increased in the presence of the test compound in the second myocyte more than in the first myocyte, then the test compound is an antagonist of at least one (non-$P2X_4$ or -$P2X_6$) P2 purinoceptor encoded by the introduced nucleic acid of the second myocyte.

The invention also includes a recombinant myocyte comprising at least one introduced nucleic acid encoding at least one P2 purinoceptor. The introduced nucleic acid is constructed such that it is expressed in the myocyte. Innumerable nucleic acid expression constructs are known in the art, and the introduced nucleic acid(s) may comprise essentially any such construct. The P2 purinoceptor encoded by the recombinant gene may be a purinoceptor encoded by a nucleic acid normally contained within a cell of the animal or may be a purinoceptor encoded by a nucleic acid obtained from a different animal, such as a human. In either case, the myocyte

comprising the introduced nucleic acid expresses this P2 purinoceptor at a higher level than the same myocyte not comprising the introduced nucleic acid. The myocyte may comprise one or more introduced nucleic acid, each encoding the same or different P2 purinoceptors.

The myocyte may be one obtained from any animal or may be cultured from an embryo of any animal. The animal is preferably a mammal, such as a rat or a human, or an animal from which myocytes may be easily obtained, such as a chick. The myocyte is preferably a cardiac myocyte such as an adult rat ventricular myocyte or a ventricular myocyte obtained by culturing a ventricular cell obtained from a chick embryo as described herein.

The P2 purinoceptor may be any P2 purinoceptor, and is preferably a P2X purinoceptor or a P2Y purinoceptor. Contemplated P2X receptors include, for example, a $P2X_1$ receptor, a $P2X_2$ receptor, a $P2X_3$ receptor, a $P2X_4$ receptor, a $P2X_5$ receptor, and a $P2X_6$ receptor. Preferably, the P2X purinoceptor is a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, or a $P2X_4/P2X_6$ heterodimer. The P2 purinoceptor may be a normal component of the myocyte which is expressed at a higher level when the myocyte comprises the introduced nucleic acid, or the P2 purinoceptor may be one which is expressed by the myocyte only when the myocyte comprises the introduced nucleic acid. A $P2X_4/P2X_6$ heterodimer may be expressed by a myocyte when the cell expresses both $P2X_4$ and $P2X_6$ purinoceptors. Preferably, the purinoceptor is a mammalian purinoceptor, more preferably a human purinoceptor.

The recombinant myocyte may be made by preparing a nucleic acid vector comprising the introduced nucleic acid and delivering the nucleic acid vector to an animal myocyte. Any known method of preparing a nucleic acid vector and any known method of transforming or transfecting an animal cell using the vector may be used. By way of example, the vector may be the plasmid designated pcDNA3, the recombinant gene may be incorporated into the plasmid using standard molecular biology techniques, and a chick embryonic ventricular myocyte may be transfected

using the plasmid by performing the modified calcium phosphate transfection method described by Xu et al., (1992, Nucl. Acids Res. 20:6425-6426).

The invention also includes a kit for determining whether a compound enhances cardiac contractility. In one embodiment, the kit comprises a recombinant myocyte comprising an introduced nucleic acid which encodes a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, or both. The kit further comprises an instructional material, as described herein, which describes assessing the contractile amplitude of the myocyte or assessing calcium uptake by the myocyte. By culturing the myocyte in the presence and absence of the compound and assessing the contractile amplitude of the myocyte, or assessing calcium uptake by the myocyte, one may determine whether the compound is an agonist of the purinoceptor(s), an antagonist of the purinoceptor(s), or neither.

In another embodiment of the kit of the invention, the kit comprises a first myocyte comprising a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, or both, and a second recombinant myocyte comprising the $P2X_4$ purinoceptor, the $P2X_6$ purinoceptor, or both, and further comprising at least one introduced nucleic acid encoding at least one second P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. The first myocyte may be recombinant or not, and the second myocyte is preferably of the same type as the first. The kit may further include an instructional material which describes assessment of the contractile amplitude of the myocytes. By comparing the change in the contractile amplitude of the first myocyte upon exposure to a test compound with the change in the contractile amplitude of the second myocyte upon exposure to the test compound, one may determine whether the test compound is an agonist, an antagonist, or neither, of the second P2 purinoceptor(s). If the test compound is an agonist of the second P2 purinoceptor(s), then the increase in the contractile amplitude of the second myocyte upon exposure to the test compound will be greater than the increase in the contractile amplitude of the first myocyte upon exposure to the test compound. If the test compound is an antagonist of the second P2 purinoceptor(s), then the increase in the contractile amplitude of the second myocyte

upon exposure to the test compound will be less than the increase in the contractile amplitude of the first myocyte upon exposure to the test compound.

Agonists of $P2X_4$ and $P2X_6$ purinoceptors are useful for treatment of any diseases associated with sub-normal myocyte contractility, such as heart failure. Likewise, antagonists of P2 purinoceptors other than $P2X_4$ and $P2X_6$ purinoceptors are useful for treatment of any diseases associated with sub-normal myocyte contractility. Such diseases include, but are not limited to heart attack or myocardial infarction, myocarditis, and various cardiomyopathies. Thus, given the disclosure provided herein, it is possible to augment cardiac contractility in an animal by administering to the animal a composition comprising a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, an antagonist of a purinoceptor other than $P2X_4$ and $P2X_6$, or some combination of these agonists and antagonists. Such a composition may, of course, further comprise a pharmaceutically acceptable carrier, particularly where the composition is intended for administration to humans.

The invention encompasses the preparation and use of medicaments or pharmaceutical compositions comprising a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, an antagonist of a purinoceptor other than $P2X_4$ and $P2X_6$, or some combination of these agonists and antagonists as an active ingredient(s). Such a pharmaceutical composition may consist of the active ingredient(s) alone, in a form suitable for administration to a subject, or the pharmaceutical composition may comprise the active ingredient(s) and one or more pharmaceutically acceptable carriers, one or more additional ingredients, or some combination of these. Administration of one of these pharmaceutical compositions to a subject is useful for enhancing cardiac contractility in the subject, as described elsewhere in the present disclosure. The active ingredient(s) may be present in the pharmaceutical composition in the form of a physiologically acceptable ester or salt, such as in combination with a physiologically acceptable cation or anion, as is well known in the art.

As used herein, the term "pharmaceutically acceptable carrier" means a chemical composition with which the active ingredient(s) may be combined and which,

following the combination, can be used to administer the active ingredient(s) to a subject, and which is not deleterious to the subject.

As used herein, the term "physiologically acceptable" ester or salt means an ester or salt form of the active ingredient(s) which is compatible with any other ingredients of the pharmaceutical composition and which is not deleterious to the subject to which the composition is to be administered.

The formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient(s) into association with a carrier or one or more other accessory ingredients, and then, if necessary or desirable, shaping or packaging the product into a desired single- or multi-dose unit.

Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for ethical administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to animals of all sorts. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions of the invention is contemplated include, but are not limited to, humans and other primates, mammals including commercially relevant mammals such as cattle, pigs, horses, sheep, cats, and dogs, birds including commercially relevant birds such as chickens, ducks, geese, and turkeys, fish including farm-raised fish and aquarium fish, and crustaceans such as farm-raised shellfish.

Pharmaceutical compositions that are useful in the methods of the invention may be prepared, packaged, or sold in formulations suitable for oral, rectal, vaginal, parenteral, topical, pulmonary, intranasal, buccal, ophthalmic, or another route

of administration which can be used to deliver a P2 purinoceptor agonist or antagonist of the invention to a cardiac myocardial tissue. Other contemplated formulations include projected nanoparticles, liposomal preparations, resealed erythrocytes containing the active ingredient(s), and immunologically-based formulations.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient(s). The amount of the active ingredient is generally equal to the dosage of the active ingredient(s) which would be administered to a subject or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

The relative amounts of the active ingredient(s), the pharmaceutically acceptable carrier, and any additional ingredients in a pharmaceutical composition of the invention will vary, depending upon the identity, size, and condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100% (w/w) active ingredient(s).

In addition to the active ingredient(s), a pharmaceutical composition of the invention may further comprise one or more additional pharmaceutically active agents.

Controlled- or sustained-release formulations of a pharmaceutical composition of the invention may be made using conventional technology.

A formulation of a pharmaceutical composition of the invention suitable for oral administration may be prepared, packaged, or sold in the form of a discrete solid dose unit including, but not limited to, a tablet, a hard or soft capsule, a cachet, a troche, or a lozenge, each containing a predetermined amount of the active ingredient(s). Other formulations suitable for oral administration include, but are not limited to, a powdered or granular formulation, an aqueous or oily suspension, an aqueous or oily solution, or an emulsion.

As used herein, an "oily" liquid is one which comprises a carbon-containing liquid molecule and which exhibits a less polar character than water.

A tablet comprising the active ingredient(s) may, for example, be made by compressing or molding the active ingredient(s), optionally with one or more additional ingredients. Compressed tablets may be prepared by compressing, in a suitable device, the active ingredient(s) in a free-flowing form such as a powder or granular preparation, optionally mixed with one or more of a binder, a lubricant, an excipient, a surface active agent, and a dispersing agent. Molded tablets may be made by molding, in a suitable device, a mixture of the active ingredient(s), a pharmaceutically acceptable carrier, and at least sufficient liquid to moisten the mixture. Pharmaceutically acceptable excipients used in the manufacture of tablets include, but are not limited to, inert diluents, granulating and disintegrating agents, binding agents, and lubricating agents. Known dispersing agents include, but are not limited to, potato starch and sodium starch glycolate. Known surface active agents include, but are not limited to, sodium lauryl sulphate. Known diluents include, but are not limited to, calcium carbonate, sodium carbonate, lactose, microcrystalline cellulose, calcium phosphate, calcium hydrogen phosphate, and sodium phosphate. Known granulating and disintegrating agents include, but are not limited to, corn starch and alginic acid. Known binding agents include, but are not limited to, gelatin, acacia, pre-gelatinized maize starch, polyvinylpyrrolidone, and hydroxypropyl methylcellulose. Known lubricating agents include, but are not limited to, magnesium stearate, stearic acid, silica, and talc.

Tablets may be non-coated or they may be coated using known methods to achieve delayed disintegration in the gastrointestinal tract of a subject, thereby providing sustained release and absorption of the active ingredient(s). By way of example, a material such as glyceryl monostearate or glyceryl distearate may be used to coat tablets. Further by way of example, tablets may be coated using methods described in U.S. Patents numbers 4,256,108; 4,160,452; and 4,265,874 to form osmotically-controlled release tablets. Tablets may further comprise a sweetening

agent, a flavoring agent, a coloring agent, a preservative, or some combination of these in order to provide pharmaceutically elegant and palatable preparation.

Hard capsules comprising the active ingredient(s) may be made using a physiologically degradable composition, such as gelatin. Such hard capsules comprise the active ingredient(s), and may further comprise additional ingredients including, for example, an inert solid diluent such as calcium carbonate, calcium phosphate, or kaolin.

Soft gelatin capsules comprising the active ingredient(s) may be made using a physiologically degradable composition, such as gelatin. Such soft capsules comprise the active ingredient(s), which may be mixed with water or an oil medium such as peanut oil, liquid paraffin, or olive oil.

Liquid formulations of a pharmaceutical composition of the invention which are suitable for oral administration may be prepared, packaged, and sold either in liquid form or in the form of a dry product intended for reconstitution with water or another suitable vehicle prior to use.

Liquid suspensions may be prepared using conventional methods to achieve suspension of the active ingredient(s) in an aqueous or oily vehicle. Aqueous vehicles include, for example, water and isotonic saline. Oily vehicles include, for example, almond oil, oily esters, ethyl alcohol, vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin. Liquid suspensions may further comprise one or more additional ingredients including, but not limited to, suspending agents, dispersing or wetting agents, emulsifying agents, demulcents, preservatives, buffers, salts, flavorings, coloring agents, and sweetening agents. Oily suspensions may further comprise a thickening agent. Known suspending agents include, but are not limited to, sorbitol syrup, hydrogenated edible fats, sodium alginate, polyvinylpyrrolidone, gum tragacanth, gum acacia, and cellulose derivatives such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose. Known dispersing or wetting agents include, but are not limited to, naturally-occurring phosphatides such as lecithin,

condensation products of an alkylene oxide with a fatty acid, with a long chain aliphatic alcohol, with a partial ester derived from a fatty acid and a hexitol, or with a partial ester derived from a fatty acid and a hexitol anhydride (e.g. polyoxyethylene stearate, heptadecaethyleneoxycetanol, polyoxyethylene sorbitol monooleate, and polyoxyethylene sorbitan monooleate, respectively). Known emulsifying agents include, but are not limited to, lecithin and acacia. Known preservatives include, but are not limited to, methyl, ethyl, or n-propyl-para- hydroxybenzoates, ascorbic acid, and sorbic acid. Known sweetening agents include, for example, glycerol, propylene glycol, sorbitol, sucrose, and saccharin. Known thickening agents for oily suspensions include, for example, beeswax, hard paraffin, and cetyl alcohol.

Liquid solutions of the active ingredient(s) in aqueous or oily solvents may be prepared in substantially the same manner as liquid suspensions, the primary difference being that the active ingredient(s) is dissolved, rather than suspended in the solvent. Liquid solutions of the pharmaceutical composition of the invention may comprise each of the components described with regard to liquid suspensions, it being understood that suspending agents will not necessarily aid dissolution of the active ingredient(s) in the solvent. Aqueous solvents include, for example, water and isotonic saline. Oily solvents include, for example, almond oil, oily esters, ethyl alcohol, vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin.

Powdered and granular formulations of a pharmaceutical preparation of the invention may be prepared using known methods. Such formulations may be administered directly to a subject, used, for example, to form tablets, to fill capsules, or to prepare an aqueous or oily suspension or solution by addition of an aqueous or oily vehicle thereto. Each of these formulations may further comprise one or more of dispersing or wetting agent, a suspending agent, and a preservative. Additional excipients, such as fillers and sweetening, flavoring, or coloring agents, may also be included in these formulations.

A pharmaceutical composition of the invention may also be prepared, packaged, or sold in the form of oil-in-water emulsion or a water-in-oil emulsion. The oily phase may be a vegetable oil such as olive or arachis oil, a mineral oil such as liquid paraffin, or a combination of these. Such compositions may further comprise one or more emulsifying agents such as naturally occurring gums such as gum acacia or gum tragacanth, naturally-occurring phosphatides such as soybean or lecithin phosphatide, esters or partial esters derived from combinations of fatty acids and hexitol anhydrides such as sorbitan monooleate, and condensation products of such partial esters with ethylene oxide such as polyoxyethylene sorbitan monooleate. These emulsions may also contain additional ingredients including, for example, sweetening or flavoring agents.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for rectal administration. Such a composition may be in the form of, for example, a suppository, a retention enema preparation, and a solution for rectal or colonic irrigation.

Suppository formulations may be made by combining the active ingredient(s) with a non-irritating pharmaceutically acceptable excipient which is solid at ordinary room temperature (i.e. about 20°C) and which is liquid at the rectal temperature of the subject (i.e. about 37°C in a healthy human). Suitable pharmaceutically acceptable excipients include, but are not limited to, cocoa butter, polyethylene glycols, and various glycerides. Suppository formulations may further comprise various additional ingredients including, but not limited to, antioxidants and preservatives.

Retention enema preparations or solutions for rectal or colonic irrigation may be made by combining the active ingredient(s) with a pharmaceutically acceptable liquid carrier. As is well known in the art, enema preparations may be administered using, and may be packaged within, a delivery device adapted to the rectal anatomy of the subject. Enema preparations may further comprise various additional ingredients including, but not limited to, antioxidants and preservatives.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for vaginal administration. Such a composition may be in the form of, for example, a suppository, an impregnated or coated vaginally-insertable material such as a tampon, a douche preparation, or a solution for vaginal irrigation.

Methods for impregnating or coating a material with a chemical composition are known in the art, and include, but are not limited to methods of depositing or binding a chemical composition onto a surface, methods of incorporating a chemical composition into the structure of a material during the synthesis of the material (i.e. such as with a physiologically degradable material), and methods of absorbing an aqueous or oily solution or suspension into an absorbent material, with or without subsequent drying.

Douche preparations or solutions for vaginal irrigation may be made by combining the active ingredient(s) with a pharmaceutically acceptable liquid carrier. As is well known in the art, douche preparations may be administered using, and may be packaged within, a delivery device adapted to the vaginal anatomy of the subject. Douche preparations may further comprise various additional ingredients including, but not limited to, antioxidants, antibiotics, antifungal agents, and preservatives.

As used herein, "parenteral administration" of a pharmaceutical composition includes any route of administration characterized by physical breaching of a tissue of a subject and administration of the pharmaceutical composition through the breach in the tissue. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, subcutaneous, intraperitoneal, intravenous, intraarterial, intramuscular, or intrasternal injection and intravenous, intraarterial, or kidney dialytic infusion techniques.

Formulations of a pharmaceutical composition suitable for parenteral administration comprise the active ingredient(s) combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules or in multi-dose containers containing a preservative. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for parenteral administration, the active ingredient(s) is provided in dry (i.e. powder or granular) form for reconstitution with a suitable vehicle (e.g. sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition.

The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient(s), additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations may be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations which are useful include those which comprise the active ingredient(s) in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer systems. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

Formulations suitable for topical administration include, but are not limited to, liquid or semi-liquid preparations such as liniments, lotions, oil-in-water or water-in-oil emulsions such as creams, ointments or pastes, and solutions or suspensions. Topically-administrable formulations may, for example, comprise from about 1% to about 10% (w/w) active ingredient(s), although the concentration of the active ingredient(s) may be as high as the solubility limit of the active ingredient(s) in the solvent. Formulations for topical administration may further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for pulmonary administration via the buccal cavity. Such a formulation may comprise dry particles which comprise the active ingredient(s) and which have a diameter in the range from about 0.5 to about 7 nanometers, and preferably from about 1 to about 6 nanometers. Such compositions are conveniently in the form of dry powders for administration using a device comprising a dry powder reservoir to which a stream of propellant may be directed to disperse the powder or using a self-propelling solvent/powder-dispensing container such as a device comprising the active ingredient(s) dissolved or suspended in a low-boiling propellant in a sealed container. Preferably, such powders comprise particles wherein at least 98% of the particles by weight have a diameter greater than 0.5 nanometers and at least 95% of the particles by number have a diameter less than 7 nanometers. More preferably, at least 95% of the particles by weight have a diameter greater than 1 nanometer and at least 90% of the particles by number have a diameter less than 6 nanometers. Dry powder compositions preferably include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form.

Low boiling propellants generally include liquid propellants having a boiling point of below 65°F at atmospheric pressure. Generally the propellant may constitute 50 to 99.9% (w/w) of the composition, and the active ingredient(s) may constitute 0.1 to 20% (w/w) of the composition. The propellant may further comprise additional ingredients such as a liquid non-ionic or solid anionic surfactant or a solid

diluent (preferably having a particle size of the same order as particles comprising the active ingredient(s)).

Pharmaceutical compositions of the invention formulated for pulmonary delivery may also provide the active ingredient(s) in the form of droplets of a solution or suspension. Such formulations may be prepared, packaged, or sold as aqueous or dilute alcoholic solutions or suspensions, optionally sterile, comprising the active ingredient(s), and may conveniently be administered using any nebulization or atomization device. Such formulations may further comprise one or more additional ingredients including, but not limited to, a flavoring agent such as saccharin sodium, a volatile oil, a buffering agent, a surface active agent, or a preservative such as methylhydroxybenzoate. The droplets provided by this route of administration preferably have an average diameter in the range from about 0.1 to about 200 nanometers.

The formulations described herein as being useful for pulmonary delivery are also useful for intranasal delivery of a pharmaceutical composition of the invention.

Another formulation suitable for intranasal administration is a coarse powder comprising the active ingredient(s) and having an average particle from about 0.2 to 500 micrometers. Such a formulation is administered in the manner in which snuff is taken i.e. by rapid inhalation through the nasal passage from a container of the powder held close to the nares.

Formulations suitable for nasal administration may, for example, comprise from about as little as 0.1% (w/w) and as much as 100% (w/w) of the active ingredient(s), and may further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for buccal administration. Such formulations may, for example, be in the form of tablets or lozenges made using conventional methods, and may, for example, 0.1 to 20% (w/w) active ingredient(s),

the balance comprising an orally dissolvable or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for buccal administration may comprise a powder or an aerosolized or atomized solution or suspension comprising the active ingredient(s). Such powdered, aerosolized, or aerosolized formulations, when dispersed, preferably have an average particle or droplet size in the range from about 0.1 to about 200 nanometers, and may further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for ophthalmic administration. Such formulations may, for example, be in the form of eye drops including, for example, a 0.1-1.0% (w/w) solution or suspension of the active ingredient(s) in an aqueous or oily liquid carrier. Such drops may further comprise buffering agents, salts, or one or more other of the additional ingredients described herein. Other ophthalmalmically-administrable formulations which are useful include those which comprise the active ingredient(s) in microcrystalline form or in a liposomal preparation.

As used herein, "additional ingredients" include, but are not limited to, one or more of the following: excipients; surface active agents; dispersing agents; inert diluents; granulating and disintegrating agents; binding agents; lubricating agents; sweetening agents; flavoring agents; coloring agents; preservatives; physiologically degradable compositions such as gelatin; aqueous vehicles and solvents; oily vehicles and solvents; suspending agents; dispersing or wetting agents; emulsifying agents, demulcents; buffers; salts; thickening agents; fillers; emulsifying agents; antioxidants; antibiotics; antifungal agents; stabilizing agents; and pharmaceutically acceptable polymeric or hydrophobic materials. Other "additional ingredients" which may be included in the pharmaceutical compositions of the invention are known in the art and described, for example in Genaro, ed., 1985, Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, which is incorporated herein by reference.

A pharmaceutical composition of the invention may be administered to deliver a dose of between 1 ng/kg/day and 100 mg/kg/day, and preferably to deliver an amount of a $P2X_4$ or $P2X_6$ agonist sufficient to effect in a cardiac tissue of the subject an agonist concentration from at least about the EC_{50} value *in vitro* to ten times, or even one hundred times, that value. Alternately, or in addition, the pharmaceutical composition of the invention may be administered to deliver a dose of between 1 ng/kg/day and 100 mg/kg/day, and preferably to deliver an amount of an antagonist of a P2 receptor other than $P2X_4$ or $P2X_6$ sufficient to effect in a cardiac tissue of the subject an antagonist concentration from at least about the IC_{50} value *in vitro* to ten times, or even one hundred times, that value. The EC_{50} value of a $P2X_4$ or $P2X_6$ agonist is the concentration of the agonist which effects a half-maximal change in the contractility of a myocyte, and may be readily determined by the ordinarily skilled artisan in view of the present disclosure. Similarly, the IC_{50} value of an antagonist of a P2 receptor other than $P2X_4$ or $P2X_6$ is the concentration of the antagonist which effects a half-maximal change in the contractility of a myocyte comprising a $P2X_4$, $P2X_6$, or both, receptors, and may be readily determined by the ordinarily skilled artisan in view of the present disclosure. When the $P2X_4$ or $P2X_6$ agonist is 2-methylthio-ATP, the agonist is preferably administered in an amount sufficient to effect a concentration of at least about 0.3 micromolar, and more preferably at least about 3 micromolar, 2-methylthio-ATP in a cardiac tissue (e.g. left or right ventricle muscle tissue) of the subject. When the antagonist of a P2 receptor other than $P2X_4$ or $P2X_6$ is suramin, reactive blue-2, or PPADS, the antagonist is preferably administered in an amount sufficient to effect a concentration of at least about 30 micromolar, and more preferably at least about 300 micromolar, antagonist in a cardiac tissue of the subject

It is understood that the ordinarily skilled physician or veterinarian will readily determine and prescribe an effective amount of the compound to enhance cardiac contractility in the subject. In so proceeding, the physician or veterinarian may, for example, prescribe a relatively low dose at first, subsequently increasing the dose

until an appropriate response is obtained. It is further understood, however, that the specific dose level for any particular subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the severity of the myocardial condition (e.g. heart failure) being treated. Furthermore, determination of an appropriate amount of an agonist or antagonist to administer to a subject by way of any particular route of administration in order to achieve the effective amounts of the agonists and antagonists described herein involves application of standard pharmacological principles and experience which may be performed by the ordinarily skilled artisan in view of the present disclosure.

Another aspect of the invention relates to a kit comprising one or more pharmaceutical compositions of the invention. In one aspect, the kit comprises a first pharmaceutical composition comprising an agonist of a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, or both, and a second pharmaceutical composition comprising at least one antagonist of at least one purinoceptor other than a $P2X_4$ or $P2X_6$ purinoceptor. For example, such a kit may comprise separate vials of the two pharmaceutical compositions, or it may further comprise an instructional material which describes administration of one, the other, or both, pharmaceutical compositions to an animal, such as a human, who is experiencing heart failure.

As used herein, an "instructional material" includes a publication, a recording, a diagram, or any other medium of expression which is used to communicate, for example, the usefulness of the pharmaceutical composition of the invention for enhancing cardiac contractility in a subject. The instructional material may also, for example, describe an appropriate dose of the pharmaceutical composition of the invention. The instructional material of the kit of the invention may, for example, be affixed to a container which contains a pharmaceutical composition of the invention or be shipped together with a container which contains the pharmaceutical composition. Alternatively, the instructional material may be shipped separately from

the container with the intention that the instructional material and the pharmaceutical composition be used cooperatively by the recipient.

The kit may further comprise a delivery device for delivering a pharmaceutical composition of the invention to a subject. By way of example, the delivery device may be a squeezable spray bottle, a metered-dose spray bottle, an aerosol spray device, an atomizer, a dry powder delivery device, a self-propelling solvent/powder-dispensing device, a syringe, a needle, a tampon, or a dosage measuring container. The kit may further comprise an instructional material as described herein.

Given the disclosure provided herein, it is possible to treat an animal such as a mammal (e.g. a human) experiencing heart failure by administering to the animal a pharmaceutical composition of the invention. It will be understood that such pharmaceutical compositions may be used to enhance cardiac contractility in an animal for any reason.

Definitions

As used herein, an "introduced" nucleic acid is a nucleic acid which does not naturally occur in a cell. Examples of introduced nucleic acids include nucleic acid vectors such as plasmids, virus vectors, naked DNA and the like.

As used herein, a "recombinant" myocyte is one which comprises a nucleic acid which is not present in a naturally occurring form in the myocyte (i.e. an "introduced" nucleic acid) and which encodes at least one P2 purinoceptor. The introduced nucleic acid may, for example, be a nucleic acid encoding a protein which is naturally expressed by a species different than the species from which the myocyte was obtained. Further by way of example, the introduced nucleic acid may comprise an additional copy of a nucleic acid which naturally occurs in the myocyte (i.e. yielding a myocyte with a non-naturally occurring number of copies of the nucleic acid).

The term "non-recombinant" myocyte is given a special meaning herein, and means a myocyte which does not comprise an introduced nucleic acid which encodes a P2 purinoceptor. Non-recombinant myocytes may thus comprise one or

more introduced nucleic acids (e.g. a nucleic acid vector used for transformation of cells) which do not encode a P2 purinoceptor.

As used herein, the "contractile amplitude" of a myocyte means the geometric magnitude of contractile motion exhibited by a contractile cell such as a myocyte.

A compound "enhances" cardiac contractility if the contractile amplitude of cardiac myocytes is greater in the presence of the compound than in the absence of the compound.

As used herein, an "agonist" of a P2 purinoceptor is a compound which activates the purinoceptor.

As used herein, an "antagonist" of a P2 purinoceptor is a compound which blocks or inhibits activation of the purinoceptor by an agonist of the purinoceptor.

The invention is now described with reference to the following Experimental Example. This Experimental Example is provided for the purpose of illustration only and the invention should in no way be construed as being limited to this Experimental Example, but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Experimental Example

The invention is now described with reference to the following Experimental Example. This Example is provided for the purpose of illustration only and the invention should in no way be construed as being limited to this Example but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

ATP induces a pronounced positive inotropic effect in a cardiac ventricular myocyte. However, the P2 receptor-effector mechanism that underlies this stimulatory cardiac action was not previously well understood. The objectives of the experiments described in this Example were to develop a cultured chick embryo ventricular myocyte as a model for cardiac P2 purinoceptor and to determine the

mechanism underlying the positive inotropic effect caused by contacting the purinoceptor with ATP. ATP caused an 89 ± 8.9 % increase in myocyte contractility. The efficacy and potency order of adenine nucleotides can be represented as follows: ATP>ADP>AMP>> adenosine. 2-Methylthio-ATP (2-MeSATP) was able to stimulate myocyte contractility, causing a maximal increase of contractility of 54 ± 2.6 %. α,β-methylene-ATP was not able to stimulate myocyte contractility.

Although UTP potently stimulates phosphoinositide hydrolysis, it had only a modest positive inotropic effect, causing a 27 ± 7 % maximal increase in myocyte contractility. The positive inotropic response stimulated by 2-MeSATP does not require the activity of phospholipase C (PLC). Instead, the effect of UTP on contractility appears to be mediated via a 2-MeSATP-sensitive P2 receptor. A PLC inhibitor, designated U-73122, had no effect on the positive inotropic response stimulated by 2-MeSATP, as indicated in Figure 6. This observation provides further evidence that PLC does not mediate the inotropic effect of 2-MeSATP. A cyclic AMP-independent calcium entry-stimulating mechanism appears to underlie a direct coupling of the receptor to stimulation of myocyte contractility. This new PLC- and cAMP-independent positive inotropic mechanism represents a target for developing novel positive inotropic therapeutics.

The materials and methods used in the experiments presented in the Experimental Example are now described.

Embryonic chick eggs were obtained from Spafas Inc. (Storrs, Conn). A cAMP radioimmunoassay kit was obtained from Amersham (Arlington Heights, IL). ^{3}H-leucine, ^{3}H-myoinositol, an $InsP_3$ radioreceptor assay kit, and ^{45}Ca were obtained from Dupont (Boston, MA). Adenosine, ADP, AMP, α,β-methylene-ATP, β,γ-methylene-ATP, and UTP were obtained from Sigma Chemical Co. (St. Louis, MO). 2-MeSATP was obtained from Research Biochemical International (Natick, MA). U-73122, which is also called 1(6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5 -dione, and U-73343, which is also called

1(6-((17b-3-methoxyestra-1,3,5(10)-trien- 17-yl)amino)hexyl)-2,5-pyrrolidine-dione were obtained from BIOMOL (Plymouth Meeting, PA).

Preparation of Cultured Cardiac Cells

Ventricular cells were obtained from chick embryos and cultured 14 days *in ovo* as described (Barry et al., 1982, J. Physiol. 325:243-260; Liang et al., 1995, Circ. Res. 76:242-251). After neutralizing trypsin with a medium comprising horse serum, the ventricular cells were centrifuged and resuspended in a culture medium comprising 6% (v/v) fetal bovine serum, 40% (v/v) Medium 199 (GIBCO, Grand Island, NY), 0.1 % (w/v each) penicillin/streptomycin, and Hank's salts. The cultured ventricular cells were plated at a density of 400,000 cell per milliliter and cultivated in a humidified 1:19 (vol:vol) CO_2:air atmosphere at 37°C. Cultured cardiac myocytes grew to confluence on day 3 in culture and exhibited rhythmic spontaneous contraction.

Assessment of ^{45}Ca Uptake into Myocardial Cells and Cellular cAMP Level

Determination of ^{45}Ca uptake was performed as described (Liang et al., 1996, J. Biol. Chem. 271:18678-18685). Cultures were incubated with L-(3, 4, 5-^{3}H, N)-leucine (152.2 Curies per millimole) for 24 hours prior to ^{45}Ca uptake. Incorporation of ^{3}H-leucine into the cellular protein permitted normalization of ^{45}Ca content to milligrams of cell protein by determining ^{3}H content, using known methods. Following exposure to ^{45}Ca, cells were then washed free of ^{45}Ca by rinsing the cells four times with ice-cold buffer containing 5 millimolar HEPES buffer, 1 millimolar $CaCl_2$, 4 millimolar KCl, 0.5 millimolar $MgCl_2$ 0.5, 142 millimolar NaCl, and 1 millimolar lanthanum, at pH 7.35. This washing procedure removed more than 99% of an extracellular marker, ^{51}Cr-EDTA, and effected substantially complete removal of extracellular ^{45}Ca. Uptake of ^{45}Ca was quantitated for 90 seconds. Cells were solubilized for 2 hours in a solution comprising 1% (w/v) sodium dodecyl sulfate and 10 millimolar sodium borate. Aliquots of the solution containing solubilized cells were assayed for radioactivity and protein content. cAMP was extracted by addition of one-tenth of the aliquot volume of 1 normal HCl to the media and boiling the mixture for 10 minutes. Extracted cAMP was assayed according to a described

radioimmunoassay method (Liang et al., 1995, Circ. Res. 76:242-251, Amersham, Arlington Heights, ILL). The effect of P2 receptor agonist on cyclic AMP accumulation was determined to be linear for at least 10 minutes, after which time the concentration of cAMP was assessed in the sample.

Determination of Contractile Amplitude

Contractile amplitude of cultured cardiac myocytes was assessed using an optico-video motion detection system as described (Barry et al., 1982, J. Physiol. 325:243-260; Liang et al., 1996, J. Biol. Chem. 271:18678-18685). Myocytes were paced at 2 Hertz by field stimulation using a platinum electrode, so that the effects of contractile amplitude were minimally affected by contractile period and the like. Myocytes were exposed a perfusion medium comprising an adenine nucleotide or analog, 4 millimolar HEPES buffer, 137 millimolar NaCl, 3.6 millimolar KCl, 0.5 millimolar $MgCl_2$, 1.1 millimolar $CaCl_2$, and 5.5 millimolar glucose at pH 7.4. Assessment of contractile amplitude was performed using only one cell per coverslip. Both the basal contraction amplitude and the amplitude measured during exposure to the adenine nucleotide or analog were determined.

Measurement of Phosphoinositide Response

Inositol phosphates were determined according to the method of Berridge et al (1983, Biochem. J. 212:473-482), as modified by Barnett et al (1990, Biochem. J. 271:437-442). Myocytes were pre-incubated with 5 millicuries per milliliter ^{3}H-myo-inositol for 24 hours, washed with Dulbecco's modified Eagle's medium comprising 15 millimolar LiCl (DMEM-Li), and incubated in DMEM-Li for 10 minutes at 37°C. The myocytes were then exposed to the adenine nucleotide or adenine analog.

Following this exposure, the myocytes were subjected to extraction using 1 milliliter of a solvent comprising a 1:2:0.05 (vol:vol:vol) mixture of chloroform:methanol:HCl to remove inositol phosphates from the myocytes. The solvent comprising inositol phosphates was applied to an anion exchange column (AGx8 resin, formate form, 1 milliliter bed volume) and $InsP_1$, InsP2, and $InsP_3$ were

eluted sequentially using a solution comprising 100 millimolar formic acid and 200 millimolar ammonium formate, a solution comprising 100 millimolar formic acid and 600 millimolar ammonium formate, and a solution comprising 100 millimolar formic acid and 1 M ammonium formate, respectively. The anion exchange column was calibrated with each inositol phosphate standard to confirm complete separation of $InsP_1$, InsP2, and $InsP_3$. Recovery of each inositol phosphate was greater than 95 %.

Inositol-1,4,5-trisphosphate Radioreceptor Assay

The effect of ATP receptor agonists on the $InsP_3$ level was quantitated using an $InsP_3$ radioreceptor assay. Growth media in which ventricular cells were grown was replaced with a solution comprising HEPES buffer, 1 millimolar $CaCl_2$, 4 millimolar KCl, and 0.5 millimolar $MgCl_2$ at pH 7.35. The cells were then exposed to ATP. The reaction was terminated by addition of 0.2 volumes of ice-cold trichloroacetic acid (TCA), which was removed by extraction with a solution comprising TCTFE (1,1,2-trichloro-1,2,2-trifluoroethane)-trioctylamine. $InsP_3$ in the aqueous phase was determined by competition with ^3H-$InsP_3$ for binding to the $InsP_3$ receptor supplied as a part of a kit, as described (Dupont, Boston, MA; Liang et al., 1996, J. Biol. Chem. 271:18678-18685).

The results of the experiments described in this Example are now described.

Characterization of the Positive Inotropic Response to ATP and Adenine Nucleotides

Treatment with ATP stimulated a marked increase in the contractile amplitude of myocytes, as illustrated in Figure 8A. The concentration of ATP which induced an approximately half-maximal increase in contractile amplitude (EC_{50}) was 0.16 ± 0.1 micromolar, the maximal increase in contractile amplitude being 89 ± 8.9% at about 3 micromolar ATP, as indicated in Figure 1.

ADP also induced a significant increase in myocyte contractility, the value of EC_{50} for ADP being 0.40 ± 0.3 micromolar. However, ADP was less efficacious than ATP for increasing contractile amplitude, the maximal increase in contractile amplitude being only 47 ± 10.5% at 10 micromolar ADP.

AMP and adenosine were less effective in stimulating myocyte contractility, the corresponding maximal increases in contractile amplitude being 10 ± 4.3% and 16 ± 3.7%, respectively. These results indicate that the inotropic effect of ATP is mediated by the P2 purinoceptor, rather than the P_1 purinoceptor.

Adult rat ventricular myocytes were isolated, and the effects of suramin and PPADS on 2-MeSATP-stimulated positive inotropic responses of those myocytes were determined. As indicated in Figures 9A and 9B, the presence of 100 micromolar suramin or 100 micromolar PPADS did not significantly affect 2-MeSATP-stimulated inotropic responses of myocytes.

To characterize the subtype of P2 purinoceptor that mediates the positive inotropic response of myocytes to ATP, a number of P2 receptor subtype-selective agonists were tested. The P2 receptor agonist 2-methylthio-ATP (2-MeSATP) caused a large increase in the contractile amplitude, as illustrated in Figure 8B. The value of EC_{50} for 2-MeSATP was 0.06 ± 0.05 micromolar. α,β-methylene-ATP and β,γ-methylene-ATP, which are agonists of some of the P2X receptors, were ineffective at stimulating myocyte contractility, as illustrated by the data presented in Figure 2. UTP, which is capable of activating the UTP-sensitive P2Y receptor, had a modest stimulatory effect on myocyte contractility, the value of EC_{50} for UTP being 0.3 ± 0.1 micromolar and the maximal increase in contractile amplitude for UTP being 27 ± 7 %. These results are consistent with a role of an UTP-sensitive P2Y receptor in mediating the positive inotropic response of myocytes to treatment with ATP. Because 2-MeSATP is a potent agonist of some of the P2X receptors, such as the $P2X_2$, $P2X_4$, $P2X_5$, and $P2X_6$ subtypes, it is possible that a P2X receptor can also mediate the ATP-induced positive inotropic effect in cardiac myocytes.

Subtype of Cardiac P2 Purinoceptor Coupled to Stimulation of Phosphatidyl-Inositol Hydrolysis

Because P2Y receptors can be coupled to activation of PIP2-PLC with consequent stimulation of phosphatidyl inositol (PI) hydrolysis (Harden et al., 1995, Annu. Rev. Pharmacol. Toxicol. 35:541-579), it was determined whether a

UTP-sensitive cardiac P2Y receptor is coupled to stimulation of PIP2-PLC and whether a resulting increase in PIP2-PLC activity mediates the positive inotropic response observed in cardiac myocytes.

Treatment of cardiac myocytes with ATP caused a significant increase in the intracellular levels of $InsP_1$, InsP2, and $InsP_3$, as depicted in Figure 3A. After 30 minutes of exposure of myocytes to ATP, there was a nearly six fold (570 ± 110%) increase in the intracellular concentration of total inositol phosphates ($InsP_{1-3}$). Half of the maximal increase in inositol phosphate concentration was achieved by treating the myocytes with an ATP concentration of 15 ± 10 micromolar, as illustrated in Figure 3B. The increase in the inositol 1,4,5-trisphosphate isomer was confirmed by using an inositol 1,4,5-trisphosphate radioreceptor assay. The basal concentration of the inositol 1,4,5-trisphosphate isomer was 42 ± 6 picomoles/milligram; in the presence of ATP the concentration of the inositol 1,4,5-trisphosphate isomer was 96 ± 4 picomoles/ milligram. The increase in $InsP_3$ concentration was transient, peaking 45 seconds after ATP treatment. Treatment of cardiac myocytes with UTP was also coupled to a pronounced stimulation of inositol phosphate production. Myocytes treated with 300 micromolar UTP experienced an increase in total inositol phosphates of 500 ± 90 %, and half-maximal inositol phosphate concentration increase was experienced by cells treated with 11 ± 10 micromolar UTP, as illustrated by the data depicted in Figure 3B. Neither the ATP- nor the UTP-stimulated PI response was attenuated by prior treatment of the myocytes with 5 nanograms pertussis toxin per milliliter over 24 hours, a treatment protocol that caused complete ADP-ribosylation of Gi by endogenous NAD^+ in these cultures (Liang et al., 1995, Circ. Res. 76:242-251; Liang et al., 1996, J. Biol. Chem. 271:18678-18685).

Since ATP is a potent agonist of both P2Y and P2X receptors, it is possible that the positive inotropic effect of ATP is mediated by both a PIP2-PLC-coupled, UTP-sensitive P2Y receptor and a phospholipase C-independent P2X or P_{2Y} receptor. To test this hypothesis, the effect of UTP and 2-MeSATP on PI hydrolysis was examined. UTP was as effective as ATP in stimulating PI hydrolysis,

while 2-MeSATP caused only a small increase in PI hydrolysis (52 ± 9 %). The P2X receptor-selective agonists α,β-methylene-ATP and β,γ-methylene-ATP were ineffective in stimulating PI hydrolysis. These data are consistent with the idea that a UTP-sensitive cardiac P2Y receptor is closely coupled to the activation of PIP2-PLC, and that a separate 2-MeSATP-sensitive P2 receptor is potently coupled to stimulation of myocyte contractility but is inefficiently coupled to PIP2-PLC activity. Alternatively, a P2X or P2Y receptor, activated by 2-MeSATP, may be selectively coupled to stimulation of myocyte contractility, while the UTP-sensitive P2Y receptor is coupled only to PIP2-PLC activity, which activity has no effect on the myocyte contractility. If the latter hypothesis is correct, then the stimulatory effect of 2-MeSATP on the PLC activity is due to its agonist activity at the PLC-coupled P2Y receptor, and the positive inotropic effect of UTP is due to its agonist activity at a 2-MeSATP-sensitive P2 purinoceptor. To provide further evidence for this conclusion, a number of cross-desensitization experiments were carried out.

Role of PIP2-PLC in Mediating the P2 Receptor Agonist-induced Positive Inotropic Response

UTP- and 2-MeSATP-induced PI hydrolysis was reduced by incubating myocytes for 80 minutes in a medium comprising 100 micromolar UTP, removing the UTP, and then assessing UTP- or 2-MeSATP- induced PI hydrolysis, as depicted in Figure 4. However, incubation of myocytes with 100 micromolar 2-MeSATP for 80 minutes prior to UTP- or 2-MeSATP induction of PI hydrolysis had no effect on the basal level of inositol phosphates, $InsP_{1-3}$ levels being 9883 ± 320 units in cells which were not incubated with 2-MeSATP and 9214 ± 410 units in cells incubated with 2-MeSATP. Incubation of myocytes with 100 micromolar 2-MeSATP also had no effect on PI hydrolysis induced by addition of either UTP ($InsP_{1-3}$ levels being 45313 ± 1820 units in myocytes not incubated with 2-MeSATP and 46576 ± 1694 units in myocytes incubated with 2-MeSATP) or 2-MeSATP ($InsP_{1-3}$ levels being 15842 ± 2010 units in myocytes not incubated with 2-MeSATP and 14265 ± 1902 units in myocytes incubated with 2-MeSATP).

Next, the role of PLC-coupled P2Y receptor in mediating the positive inotropic response was examined. An 80 minute exposure of myocytes to 100 micromolar 2-MeSATP caused a significant reduction of the ATP-induced positive inotropic response, which was manifested as a 73 ± 4 % decrease in the extent of stimulation of myocyte contractility. 2-MeSATP-exposed myocytes also exhibited a diminished positive inotropic response to 2-MeSATP, and exhibited virtually no increase in myocyte contractile amplitude in response to UTP, as depicted in Figures 5A and 5B. However, a 90 minute exposure to 100 micromolar UTP had no effect on 2-MeSATP- or UTP-induced increase in myocyte contractility, as depicted in Figures 5C and 5D. These data are consistent with the hypothesis that a P2Y receptor, activated to a much greater extent by UTP than by 2-MeSATP, is coupled to stimulation of PIP2-PLC, and that a P2X or P2Y receptor activated to a much greater extent by 2-MeSATP than by UTP is coupled to stimulation of myocyte contractility.

The aminosteroid U-73122, which is a known inhibitor of the receptor-mediated activation of PLC, was used to further determine whether PLC plays a role in 2-MeSATP-stimulated increase in myocyte contractility. Neither 1 micromolar nor 10 micromolar U-73122 had an effect on the 2-MeSATP-stimulated increase in myocyte contractility, as depicted in Figure 6. 10 micromolar U-73122, a concentration known to inhibit completely the PLC-mediated PI hydrolysis (Smith et al., 1990, J. Pharmacol. Exp. Ther. 253:688-697; Thompson et al., 1991, J. Biol. Chem. 266:23856-23862), caused a slight depression of the myocyte contractility, but did not affect 2-MeSATP-induced stimulation of contractility, the increase in contractile amplitude of myocytes treated with 2-MeSATP and U-73122 being 48.4 ± 8 %, a value comparable to that observed using 2-MeSATP alone. The inactive structural analog of U-73122, designated U-73343, also had no effect on 2-MeSATP-stimulated increase in myocyte contractility.

cAMP-independent ^{45}Ca Entry Underlies the P2 Receptor-Mediated Positive Inotropic Response

ATP can induce an increase in myocyte contractile amplitude (Berridge et al., 1983, Biochem. J. 212:473-482) as well as an increase in calcium entry and an increase in the level of cytosolic calcium (Christie et al., 1992, J. Physiol. 445:369-388; DeYoung et al., 1989, Am. J. Physiol. 257(Cell Physiol. 26):C750-C758; Scamps et al., 1992, J. Gen. Physiol. 100:675-701). Both 2-MeSATP and ATP caused a pronounced increase in the transsarcolemmal uptake of ^{45}Ca. However, neither α,β-methylene-ATP, as indicated in Figure 7, nor β,γ-methylene-ATP had any significant stimulatory effect on ^{45}Ca uptake by myocytes. Neither 2-MeSATP nor ATP was able to stimulate cAMP accumulation, the basal cAMP level being 12.2 ± 2 picomoles/milligram and cAMP levels in the presence of 2-MeSATP and ATP being 13.1 ± 1.6 and 13.2 ± 1.1, respectively. No other P2 receptor agonist, including UTP, α,β-methylene-ATP, or β,γ-methylene-ATP was able to induce cAMP accumulation in myocytes. As a positive control, cells were treated with isoproterenol, and a 6.7 ± 1.4- fold increase in the level of cAMP was observed. These data indicate that a cAMP-independent calcium entry-stimulating mechanism mediates the P2 receptor agonist-induced stimulation of myocyte contractility.

Cultured chick embryo ventricular myocytes are as a useful experimental model for a number of receptor-effector systems, including the P_1 (adenosine) receptor. Because these myocytes remain stable in culture for at least three days, various interventions can be performed, such as desensitization studies and equilibration of ^{3}H-myo-inositol within the myocyte pool to enable examination of PI hydrolysis. The feasibility of preparing relatively large number of myocytes also facilitates the biochemical determination of phosphoinositide levels. The stability of the cultured myocytes enables reliable and reproducible determination of changes in contractility in response to various agonists.

Thus, the cultured ventricular myocytes described herein represent a model system in which a stable and reproducible ATP-induced positive inotropic

response facilitates full characterization of the receptor(s) involved as well as studies of the mechanism underlying the receptor(s) physiological effects and of compounds capable of interfering with that mechanism.

Adenine nucleotides caused a pronounced stimulation of the myocyte contractility with the order of efficacy being ATP > ADP > AMP >> adenosine. This observation is consistent with the hypothesis that the positive inotropic response of myocytes to adenine nucleotide treatment is mediated by a P2 purinoceptor. That the order of potency and efficacy is ATP > 2-MeSATP > UTP > (α,β-methylene-ATP or β,γ-methylene-ATP) is consistent with the hypothesis that the subtype of P2 receptor mediating the positive inotropic effect is a P2Y receptor or a 2-MeSATP-sensitive P2X receptor, such as $P2X_2$, $P2X_4$, $P2X_5$, or $P2X_6$ receptor (Burnstock et al., 1996, Drug Develop. Res., 38:67-71).

Because UTP exhibits a significant positive inotropic effect, it was possible that a UTP-sensitive P2 receptor, either one of the known P2Y receptors or a novel receptor, also mediated some of the ATP-stimulated positive inotropic effect (Burnstock et al., 1996, Drug Develop. Res., 38:67-71; Harden et al., 1995, Annu. Rev. Pharmacol. Toxicol. 35:541-579). Although the concentrations of ATP and UTP required for half maximal stimulation of PI hydrolysis were higher than those for the increase in contractile amplitude, 1 micromolar ATP or UTP was able to induce a significant increase in intracellular inositol phosphate levels (85 ± 10 % increase using ATP and 90 ± 15 % increase using UTP). Because UTP causes a pronounced increase in the level of $InsP_{1-3}$ and because $InsP_3$ is known to stimulate release of calcium from the sarcoplasmic reticulum (Vites et al., 1990, Am. J. Physiol. 258(Heart Circ. Physiol. 27):H1745-H1752; Vites et al., 1992, Am. J. Physiol. 262(Heart Circ. Physiol. 31):H268-H277), it was possible that the receptor-mediated increase in $InsP_3$ caused the positive inotropic effect observed in UTP-treated cells. Because 2-MeSATP caused only a modest stimulation of inositol phosphate production, the 2-MeSATP-sensitive P2 receptor may be coupled directly to stimulation of myocyte contractility independent of any increase in $InsP_{1-3}$ production. According to this hypothesis, ATP

induced its positive inotropic effect by activating both a PLC-coupled P2Y receptor and a 2-MeSATP-sensitive P2 receptor. Alternatively, the positive inotropic effect induced by UTP is due to its agonist activity, although modest, at the 2-MeSATP-sensitive P2 purinoceptor, and the stimulatory effect induced by 2-MeSATP on $InsP_{1-3}$ level is caused by the cross-activity or 2-MeSATP at the UTP-sensitive P2Y receptor.

Two lines of evidence support this latter hypothesis. First, treatment of myocytes with UTP prior to treatment of the myocytes with UTP or 2-MeSATP decreased stimulation of $InsP_{1-3}$ production. On the other hand, treatment of myocytes with 2-MeSATP prior to treatment of the myocytes with UTP or 2-MeSATP had no effect on stimulation of $InsP_{1-3}$ production. These observations suggest that only the UTP-sensitive P2Y receptor is coupled to inositol phosphate production. Second, incubation of myocytes with 2-MeSATP prior to treatment of the myocytes with UTP or 2-MeSATP significantly reduced the positive inotropic response otherwise induced by this treatment. Incubation of myocytes with UTP prior to treatment of the myocytes with UTP or 2-MeSATP had no effect on UTP- or 2-MeSATP-stimulated myocyte contractility. These observations suggest that only the 2-MeSATP-sensitive P2 purinoceptor is coupled to the stimulation of myocyte contractility.

The observation that ATP can stimulate PI hydrolysis in cultured chick ventricular myocytes is similar to the observations made using mouse (Yamada et al., 1992, Circ. Res. 70:477-485) and rat (Leggssyer et al., 1988, J. Physiol. 401:185-199) ventricular myocytes. The experiments presented in this Example demonstrate that the stimulatory effect of ATP is mediated via an UTP-sensitive P2Y receptor. In contrast to previous suggestions (Leggssyer et al., 1988, J. Physiol. 401:185-199), the present observations suggest that the positive inotropic response of myocytes to ATP, such as that mediated via the 2-MeSATP-sensitive P2 receptor, occurs independently of PLC activation. Formation of $InsP_3$ is not necessary to induce the 2-MeSATP-stimulated positive inotropic response. The conclusions described herein were further supported

by the observation that 1 micromolar 2-MeSATP caused a maximal positive inotropic effect, but that this concentration of 2-MeSATP had no effect on intracellular $InsP_{1-3}$ levels. Further evidence that PLC is not involved in the 2-MeSATP-inducible inotropic response was provided by observing that U-73122, a known PLC inhibitor, had no effect on 2-MeSATP-induced myocyte contractility even at an inhibitor concentration of 10 micromolar, as indicated in Figure 9C.

ATP agonists were able to cause a significant stimulation of transsarcolemmal calcium entry in myocytes. However, none of the ATP agonists used in the experiments presented herein caused an increase in cellular cAMP content. This observation is similar to that made using rat ventricular myocytes (Scamps et al., 1992, J. Gen. Physiol. 100:675-701). The order of efficacy of adenine nucleotides for stimulating calcium entry, namely ATP > 2-MeSATP > UTP > α,β-methylene-ATP, is similar to the order of efficacy of the same compounds in stimulating myocyte contractility. Thus, these data suggest that a cAMP-independent, calcium entry-stimulating mechanism underlies the 2-MeSATP-sensitive P2 purinoceptor-mediated increase in myocyte contractile amplitude.

The present observations are compatible with findings that the classical P2Y agonist, 2-MeSATP, stimulates calcium entry via both a non-selective cation channel and a L-type calcium channel (Scamps et al., 1990, Circ. Res 67:1007-1016; Scamps et al., 1994, Br. J. Pharmacol. 113:982-986) and an increase in calcium transients in rat ventricular myocytes (Bjornsson et al., 1989, Eur. J. Biochem. 186:395-404). It is unlikely that ATP-induced acidification, with consequent stimulation of cytosolic calcium level, contributes to the positive inotropic effect of ATP, because ATP-induced acidification requires the presence of a Mg-ATP complex at a concentration 100 fold higher than that necessary to cause increased myocyte contractility.

Recombinant myocytes

Recombinant myocytes were made by transfecting embryonic chick myocytes using either a pcDNA3 vector alone or a pcDNA3 vector comprising a

recombinant gene encoding the $P2X_4$ purinoceptor. The pcDNA3 vector comprising the recombinant gene was made by inserting a nucleic acid encoding the $P2X_4$ purinoceptor (Garcia-Guzman et al., 1996, FEBS Lett. 388:123-127) into the multiple cloning site of a commercially-available pcDNA3 vector (Invitrogen, Carlsbad, CA). The embryonic chick ventricular myocytes were transfected using the vector using the modified calcium phosphate transfection method described by Xu et al. (1992, Nucl. Acids Res. 20:6425-6426). As indicated in Figure 10, the presence of the recombinant gene in myocytes approximately doubled the magnitude of the 2-MeSATP-induced calcium influx. As indicated in Figure 11, the presence of the recombinant gene in myocytes also significantly increased the magnitude of contractile magnitude induced by treatment of the myocytes with 2-MeSATP. These observations indicate that the $P2X_4$ purinoceptor is a purinoceptor which mediates induction of contractility of myocytes by adenine nucleotides such as ATP and 2-MeSATP.

Identity of Relevant P2 Purinoceptors

The identity of P2 purinoceptors that mediate the increase in myocyte contractility is now described. The facts that some of the recently cloned P2X receptors are potently activated by 2-MeSATP and that mRNAs encoding these receptors are expressed in the heart (Garcia-Guzman et al., 1996, FEBS Lett. 388:123-127; Soto et al., 1996, Proc. Natl. Acad. Sci. USA 93:3684-3688) support the conclusion that a P2X receptor mediates the positive inotropic effect of ATP. The P2X receptor is unlikely to be either the $P2X_1$ receptor or the $P2X_3$ receptor because the current mediated by the $P2X_1$ and $P2X_3$ receptors desensitizes completely within a few seconds (Collo et al., 1996, J. Neurosci. 16:2495-2507) and because these two P2X receptors can be activated by α,β-methylene-ATP.

Overall, the present data demonstrate that a novel cAMP- and PLC-independent calcium entry pathway, likely mediating the direct coupling of a P2 purinoceptor to stimulation of myocyte contractility, exists in the intact cardiac cell. The cAMP- and PLC-independent receptor is not likely to be a P2Y receptor, because the underlying positive inotropic mechanism does not involve the action of PLC or

cAMP, whereas all of the known P2Y receptors either stimulate PLC activity or inhibit adenylyl cyclase activity.

Thus, the stimulatory receptor is one or both of the $P2X_4$ and $P2X_6$ receptors. This hypothesis is supported by the observation that of the known P2X receptors, only the $P2X_4$, $P2X_5$, and $P2X_6$ receptors can be activated by 2-MeSATP. Of these three receptors, the function of only the $P2X_5$ receptor is sensitive to inhibition by suramin, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), and reactive blue-2. The functions of the $P2X_4$ and $P2X_6$ receptors are not sensitive to inhibition by these compounds. It has been discovered that the function of the P2 purinoceptor responsible for stimulating myocyte contractility is not sensitive to inhibition by suramin, PPADS, or reactive blue-2, but can be activated by 2-MeSATP. Furthermore, mRNA molecules encoding each of the $P2X_4$ and $P2X_6$ receptors are known to be expressed in the heart. Therefore, the $P2X_4$ and $P2X_6$ represent novel targets for the development of new positive inotropic therapeutics.

Cardiac ventricular myocytes were isolated from adult rats. The contractile amplitude of these myocytes was determined in the presence of both 200 nanomolar trinitrophenyl-ATP (TNP-ATP) and 3 micromolar 2-MeSATP and, separately, in the presence of 3 micromolar 2-MeSATP. TNP-ATP is an antagonist of both $P2X_1$ and $P2X_3$ purinoceptors. The contractile amplitude of the myocytes was approximately the same in the presence and absence of TNP-ATP. The fact that TNP-ATP failed to attenuate the positive inotropic response of the myocytes to 2-MeSATP indicates that the inotropic response of the myocytes to the presence of 2-MeSATP was not attributable to the $P2X_1$ purinoceptor or to the $P2X_3$ purinoceptor.

The ability of suramin, a non-selective antagonist of most P2X and P2Y purinoceptors other than $P2X_4$ to enhance the increase in contractility of adult rat and chick embryo myocytes in the presence of 2-MeSATP. This results indicates that cardiac myocyte contractility can be enhanced to a greater degree in the presence of both a $P2X_4$ (or $P2X_6$) purinoceptor agonist and an antagonist of a purinoceptor other than $P2X_4$ or $P2X_6$ than in the presence of a $P2X_4$ (or $P2X_6$) purinoceptor agonist

alone. Preferably, a $P2X_4$ purinoceptor agonist and an antagonist of a purinoceptor other than $P2X_4$ are used in combination.

Cultured chick embryo ventricular myocytes were transfected using a pcDNA3 vector comprising a nucleic acid encoding a $P2X_5$ purinoceptor. Treatment of these myocytes with 2-MeSATP (3 micromolar) did not result in greater stimulation of contractility (15 ± 4.7%) as compared with the ability of the same concentration of 2-MeSATP to stimulate contractility of cultured ventricular myocytes that had been transfected with a pcDNA3 vector which did not encode a $P2X_5$ purinoceptor (16.5 ± 1%). Cultured chick embryo ventricular myocytes transfected using a pcDNA3 vector comprising a nucleic acid encoding a $P2X_4$ purinoceptor exhibited a 45.5 ± 6% stimulation of contractility in response to 3 micromolar 2-MeSATP.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

What is claimed is:

1. A recombinant myocyte comprising at least one introduced nucleic acid encoding at least one P2 purinoceptor.

2. The recombinant myocyte of claim 1, wherein said P2 purinoceptor is selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, a $P2X_4$ purinoceptor, a $P2X_5$ purinoceptor, and a $P2X_6$ purinoceptor.

3. The recombinant myocyte of claim 1, wherein said P2 purinoceptor is selected from the group consisting of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor.

4. The recombinant myocyte of claim 1, wherein said myocyte is selected from the group consisting of a chicken embryonic ventricular myocyte and a rat ventricular myocyte.

5. A method of determining whether a compound enhances cardiac contractility, said method comprising assessing the contractile amplitude of a recombinant myocyte in the presence and absence of the compound, wherein said myocyte comprises at least one first introduced nucleic acid encoding at least one of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor, whereby an increase in the contractile amplitude of said myocyte in the presence of the compound, relative to the contractile amplitude of said myocyte in the absence of the compound, is an indication that the compound enhances cardiac contractility.

6. The method of claim 5, wherein said myocyte is selected from the group consisting of a chicken embryonic ventricular myocyte and a rat ventricular myocyte.

7. The method of claim 5, further comprising assessing the contractile amplitude of a non-recombinant myocyte in the presence and absence of the compound, whereby if the difference between the contractile amplitude of said recombinant myocyte in the presence of the compound and the contractile amplitude of said recombinant myocyte in the absence of the compound is greater than the difference between the contractile amplitude of said non-recombinant myocyte in the

presence of the compound and the contractile amplitude of said non-recombinant myocyte in the absence of the compound, then the effect of the composition on contractile amplitude is attributable to said at least one purinoceptor.

8. A method of determining whether a compound enhances cardiac contractility, said method comprising assessing calcium uptake by a recombinant myocyte in the presence and absence of the compound, wherein said myocyte comprises at least one first introduced nucleic acid encoding at least one of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor, whereby an increase in calcium uptake by said myocyte in the presence of the compound, relative to calcium uptake by said myocyte in the absence of the compound, is an indication that the compound enhances cardiac contractility.

9. The method of claim 8, further comprising assessing calcium uptake by a non-recombinant myocyte in the presence and absence of the compound, whereby if the difference between calcium uptake by said recombinant myocyte in the presence of the compound and calcium uptake by said recombinant myocyte in the absence of the compound is greater than the difference between calcium uptake by said non-recombinant myocyte in the presence of the compound and calcium uptake by said non-recombinant myocyte in the absence of the compound, then the effect of the composition on calcium uptake is attributable to said at least one purinoceptor.

10. A method of determining whether a compound enhances cardiac contractility, said method comprising

assessing the contractile amplitude of a first myocyte in the presence and absence of the compound, wherein said first myocyte comprises at least one first P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, and a $P2X_4/P2X_6$ heterodimer; and

assessing the contractile amplitude of a second myocyte in the presence and absence of the compound, wherein said second myocyte comprises said at least one first P2 purinoceptor and at least one introduced nucleic acid encoding at least one second P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor;

whereby if the difference between the contractile amplitude of said second myocyte in the presence of the compound and the contractile amplitude of said second myocyte in the absence of the compound is greater than the difference between the contractile amplitude of said first myocyte in the presence of the compound and the contractile amplitude of said first myocyte in the absence of the compound, then the compound enhances cardiac contractility.

11. The method of claim 10, wherein said second P2 purinoceptor is selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, and a $P2X_5$ purinoceptor.

12. A method of determining whether a compound enhances cardiac contractility, said method comprising

assessing calcium uptake by a first myocyte in the presence and absence of the compound, wherein said first myocyte comprises at least one first P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, and a $P2X_4/P2X_6$ heterodimer; and

assessing calcium uptake by a second myocyte in the presence and absence of the compound, wherein said second myocyte comprises said at least one first P2 purinoceptor and at least one introduced nucleic acid encoding at least one second P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor;

whereby if the difference between calcium uptake by said second myocyte in the presence of the compound and calcium uptake by said second myocyte in the absence of the compound is greater than the difference between calcium uptake by said first myocyte in the presence of the compound and calcium uptake by said first myocyte in the absence of the compound, then the compound enhances cardiac contractility.

13. The method of claim 12, wherein said second P2 purinoceptor is selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, and a $P2X_5$ purinoceptor.

14. A method of enhancing cardiac contractility in an animal, said method comprising administering to the animal a composition comprising at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, and an antagonist of a P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor, whereby cardiac contractility is augmented in the animal.

15. The method of claim 14, wherein at least one agent is selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist.

16. The method of claim 15, wherein at least one agent is 2-methylthio-ATP.

17. The method of claim 16, wherein said 2-methylthio-ATP is administered in an amount sufficient to effect a concentration of at least about 0.3 micromolar 2-methylthio-ATP in a cardiac tissue of the animal.

18. The method of claim 17, wherein said 2-methylthio-ATP is administered in an amount sufficient to effect a concentration of at least about 3 micromolar 2-methylthio-ATP in the cardiac tissue.

19. The method of claim 15, further comprising administering to the animal at least one antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

20. The method of claim 19, wherein said P2 purinoceptor is selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, and a $P2X_5$ purinoceptor.

21. The method of claim 20, wherein at least one antagonist is selected from the group consisting of suramin, reactive blue-2, and pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid.

22. The method of claim 21, wherein said at least one antagonist is administered in an amount sufficient to effect a concentration of at least about 30 micromolar antagonist in a cardiac tissue of the animal.

23. The method of claim 22, wherein said at least one antagonist is administered in an amount sufficient to effect a concentration of at least about 300 micromolar antagonist in the cardiac tissue.

24. The method of claim 14, wherein the animal is a mammal.

25. The method of claim 24, wherein the mammal is a human.

26. A pharmaceutical composition for enhancing cardiac contractility, said pharmaceutical composition comprising at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, and an antagonist of a P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

27. The pharmaceutical composition of claim 26, wherein at least one agent is selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist.

28. The pharmaceutical composition of claim 27, further comprising at least one antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

29. A kit comprising a first container containing at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist and a second container containing at least one antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

30. A method of treating an animal experiencing heart failure, said method comprising administering to the animal a composition comprising at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, and an antagonist of a P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

31. The method of claim 30, wherein at least one agent is selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist.

32. The method of claim 31, further comprising administering to the animal an antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

33. A kit for determining whether a compound enhances cardiac contractility, said kit comprising a recombinant myocyte comprising at least one introduced nucleic acid encoding at least one P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor and an instructional material which describes assessment of at least one of the contractile amplitude of said myocyte and calcium uptake by said myocyte.

34. A kit for determining whether a compound enhances cardiac contractility, said kit comprising

a first myocyte comprising at least one first P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, and a $P2X_4/P2X_6$ heterodimer; and

a second myocyte comprising at least one introduced nucleic acid encoding at least one second P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

35. A method of determining whether a compound decreases cardiac contractility, said method comprising assessing the contractile amplitude of a recombinant myocyte in the presence and absence of the compound, wherein said myocyte comprises at least one first introduced nucleic acid encoding at least one of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor, whereby a decrease in the contractile amplitude of said myocyte in the presence of the compound, relative to the contractile amplitude of said myocyte in the absence of the compound, is an indication that the compound decreases cardiac contractility.

36. The method of claim 35, further comprising assessing the contractile amplitude of a non-recombinant myocyte in the presence and absence of the compound, whereby if the decrease in the contractile amplitude of said recombinant myocyte in the presence of the compound, relative to the decrease in the contractile

amplitude of said recombinant myocyte in the absence of the compound, is greater than the decrease in the contractile amplitude of said non-recombinant myocyte in the presence of the compound, relative to the decrease in the contractile amplitude of said non-recombinant myocyte in the absence of the compound, then the effect of the composition on contractile amplitude is attributable to said at least one purinoceptor.

37. A method of decreasing cardiac contractility in an animal, said method comprising administering to the animal a composition comprising at least one agent selected from the group consisting of a $P2X_4$ purinoceptor antagonist and a $P2X_6$ purinoceptor antagonist, whereby cardiac contractility is decreased in the animal.

Fig. 1A

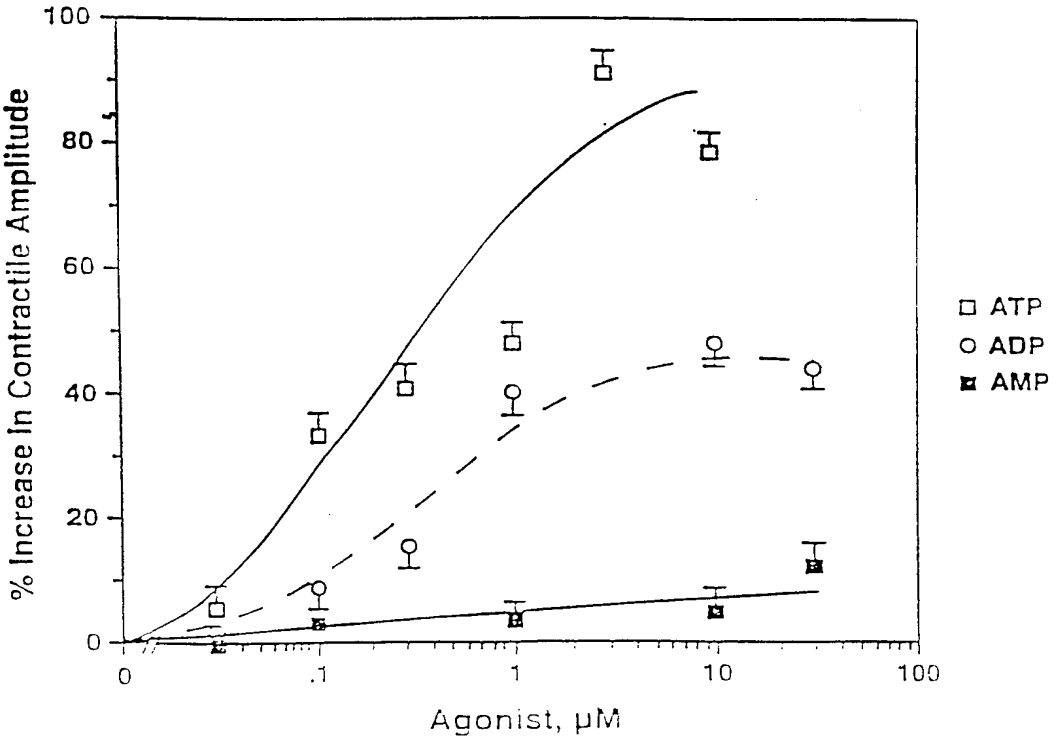

100
80
60
40
20
0
% Increase In Contractile Amplitude
0
.1
1
10
100
Agonist, µM
ATP
ADP
AMP

Fig. 1B

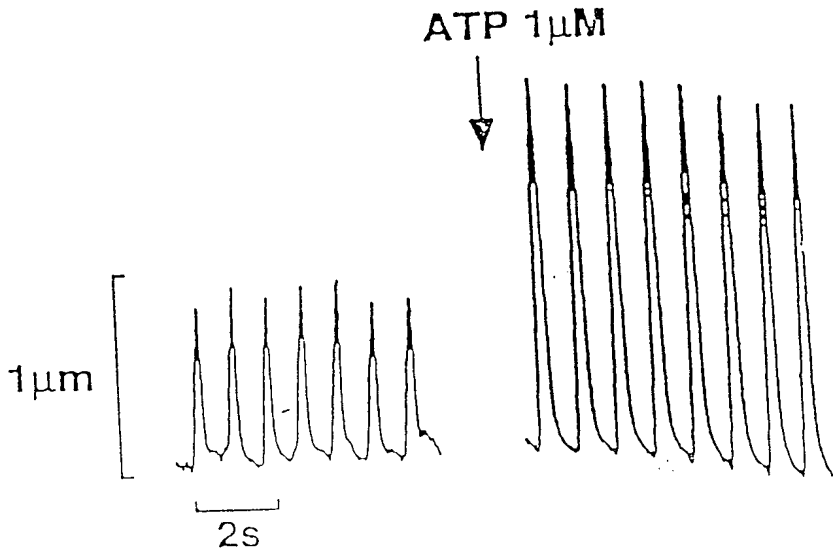


FIg-2

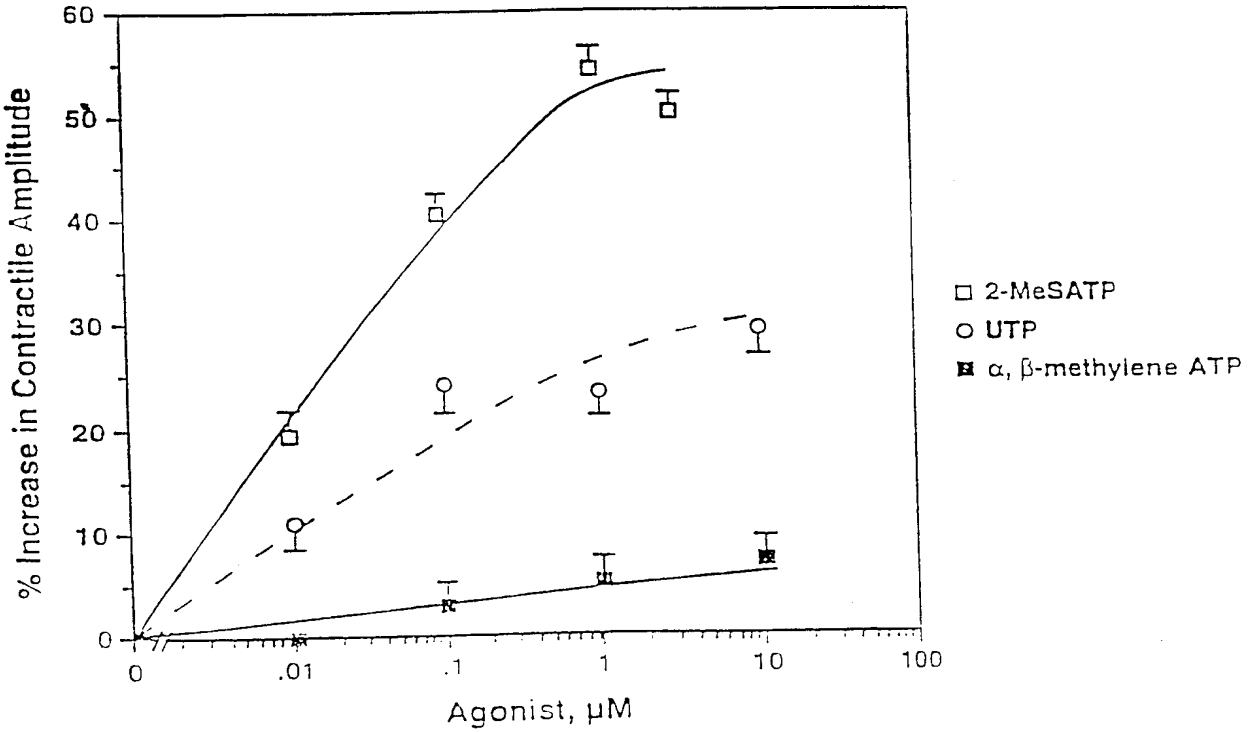
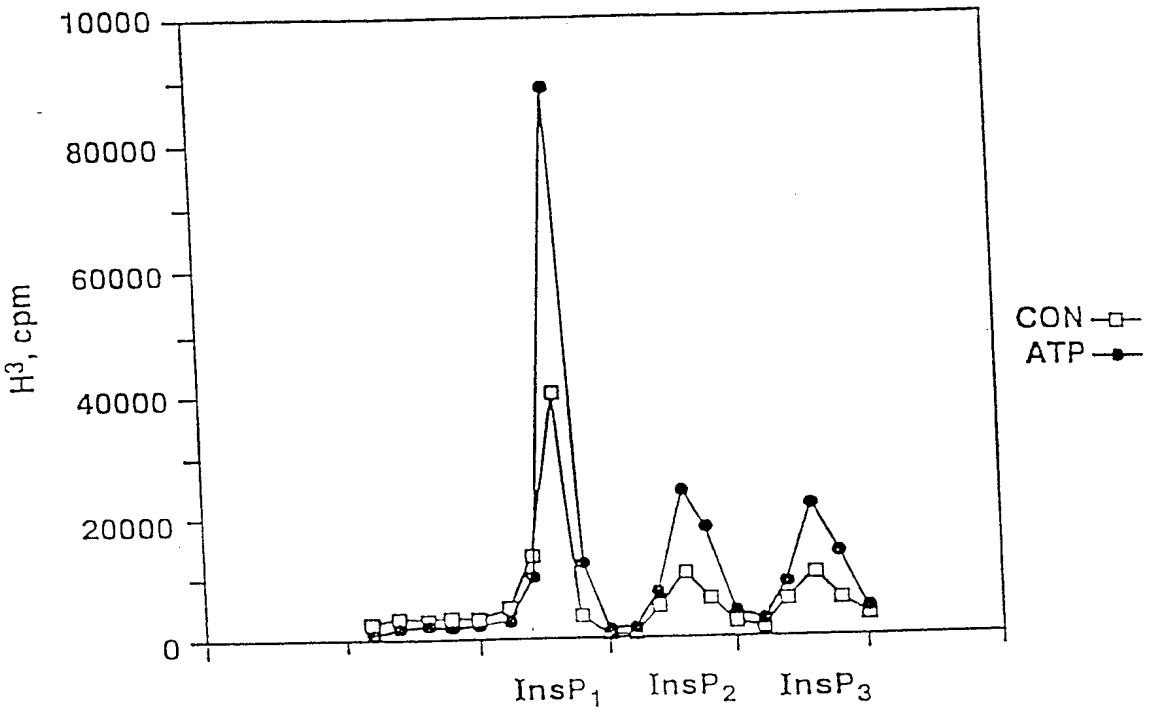

% Increase in Contractile Amplitude
60
50
40
30
20
10
0
0
.01
.1
1
10
100
Agonist, μM
□ 2-MeSATP
○ UTP
α, β-methylene ATP

Fig. 3A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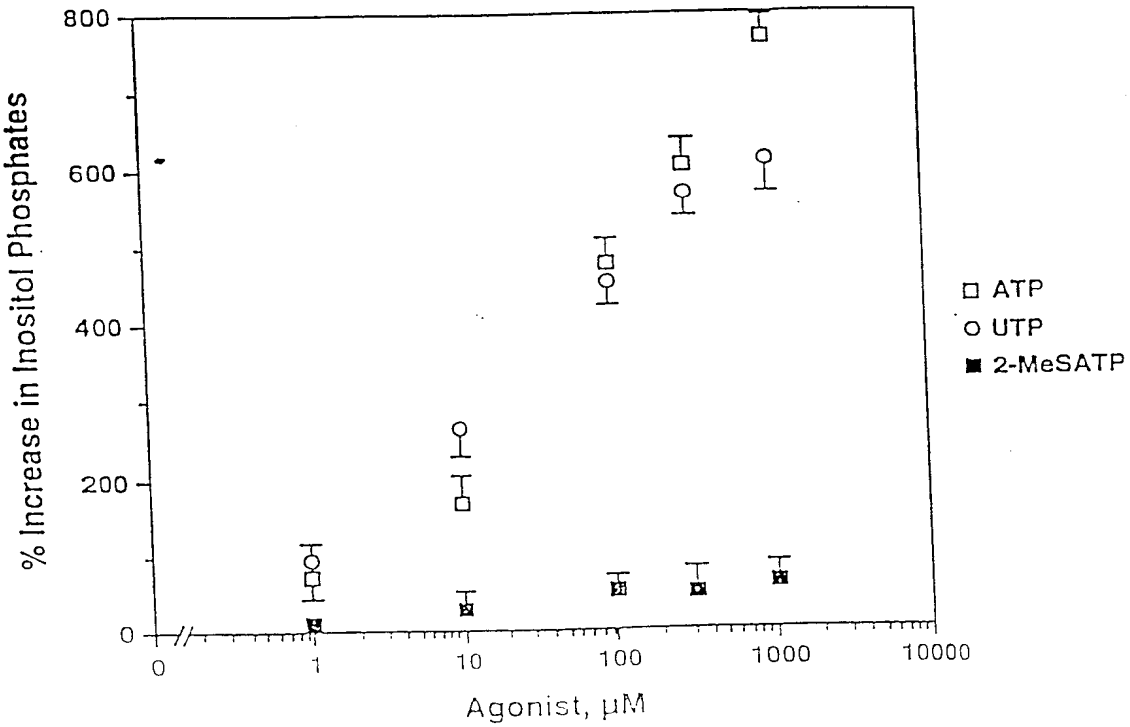
60000
40000
20000
0
H3, cpm
InsP1
InsP2
InsP3
CON
ATP

Fig. 3B


800
600
400
200
0
% Increase in Inositol Phosphates
0
1
10
100
1000
10000
Agonist, μM
ATP
UTP
2-MeSATP

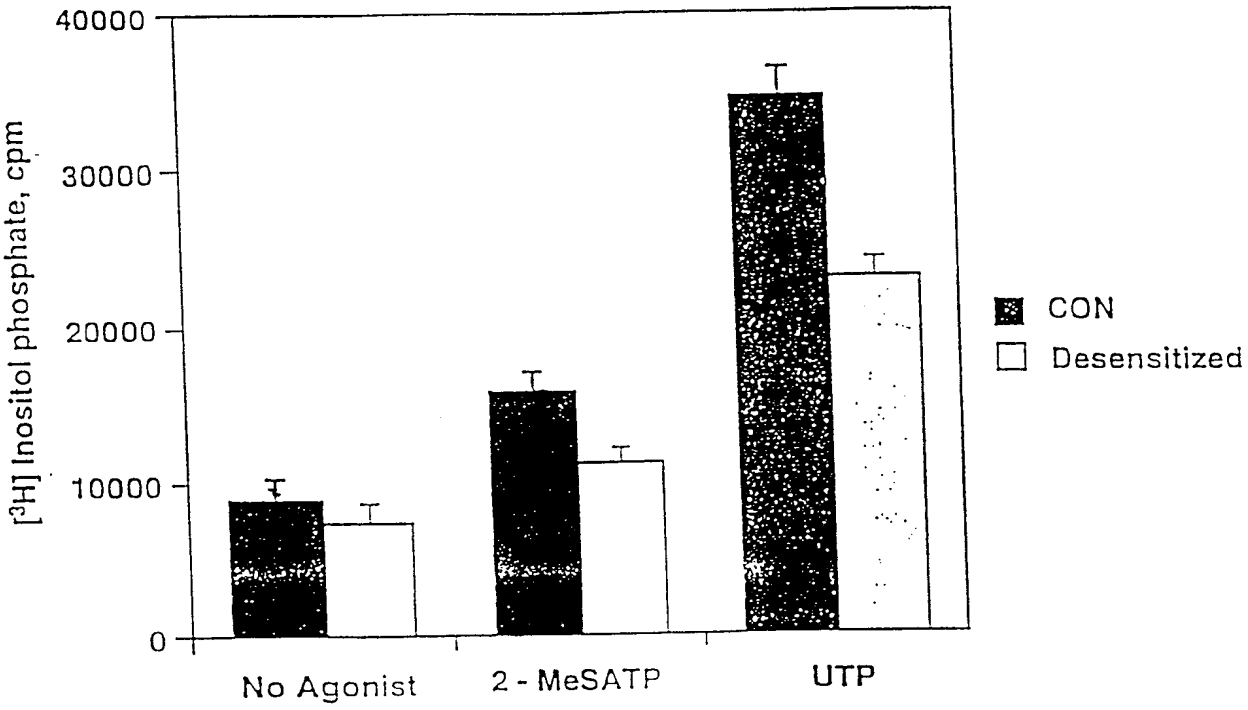

40000
30000
20000
10000
0
[3H] Inositol phosphate, cpm
No Agonist
2 - MeSATP
UTP
CON
Desensitized

Fig. 5A

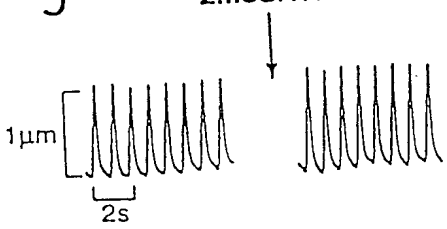


Fig. 5B

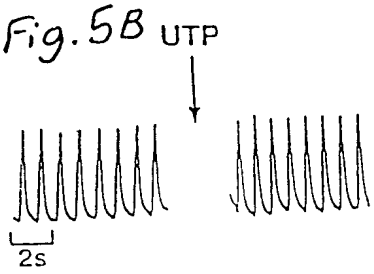


Fig. 5C

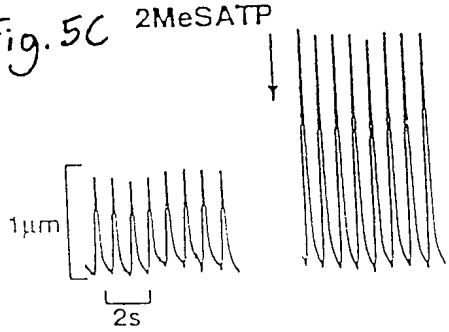


Fig. 5D

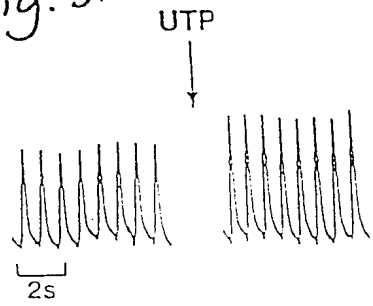


Fig. 6

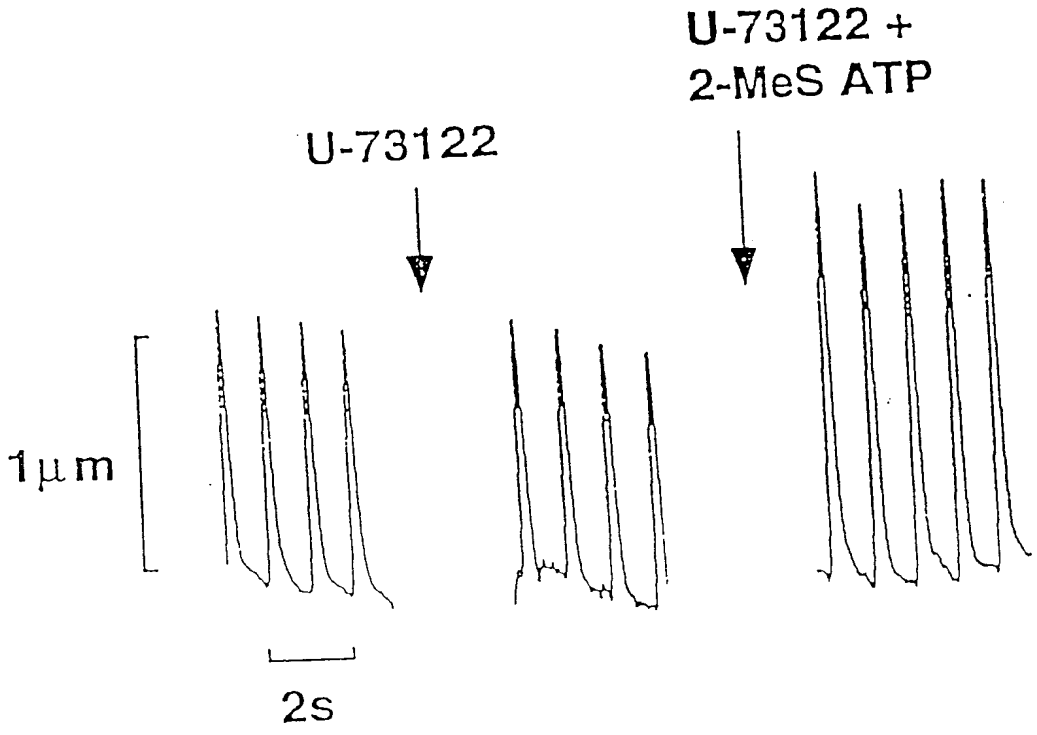
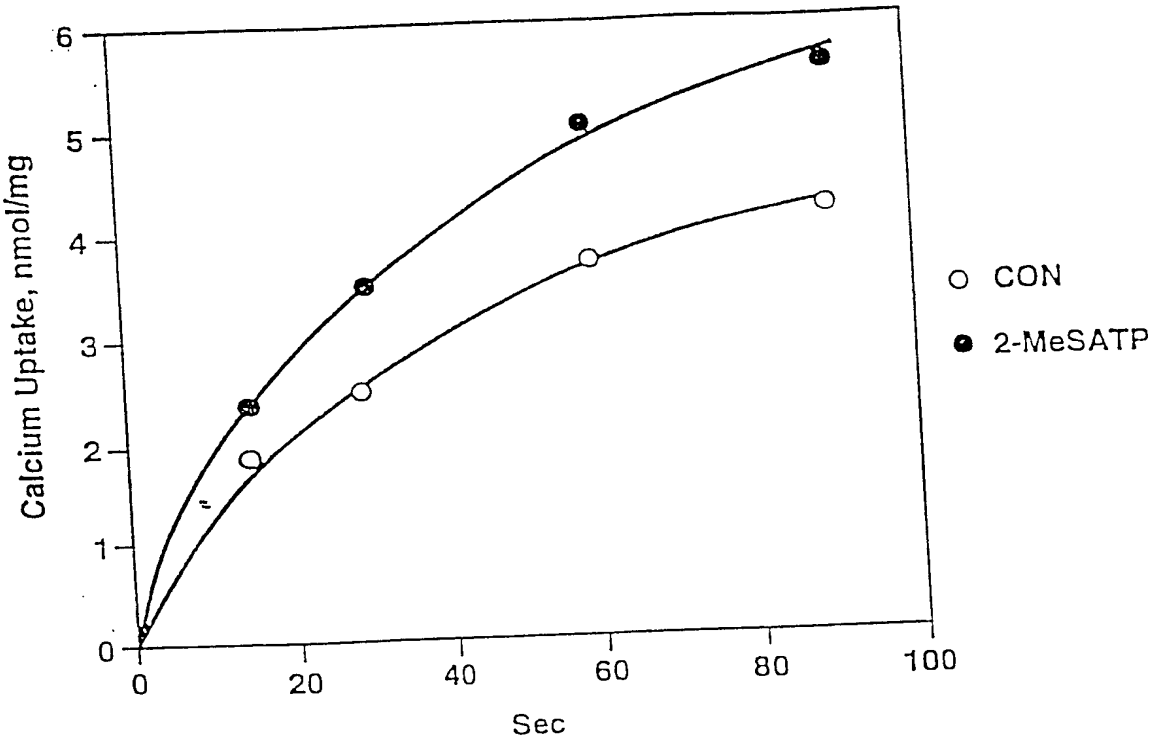


Fig. 7A


Calcium Uptake, nmol/mg
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1
2
3
4
5
6
0
20
40
60
80
100
Sec
CON
2-MeSATP

Fig. 7B

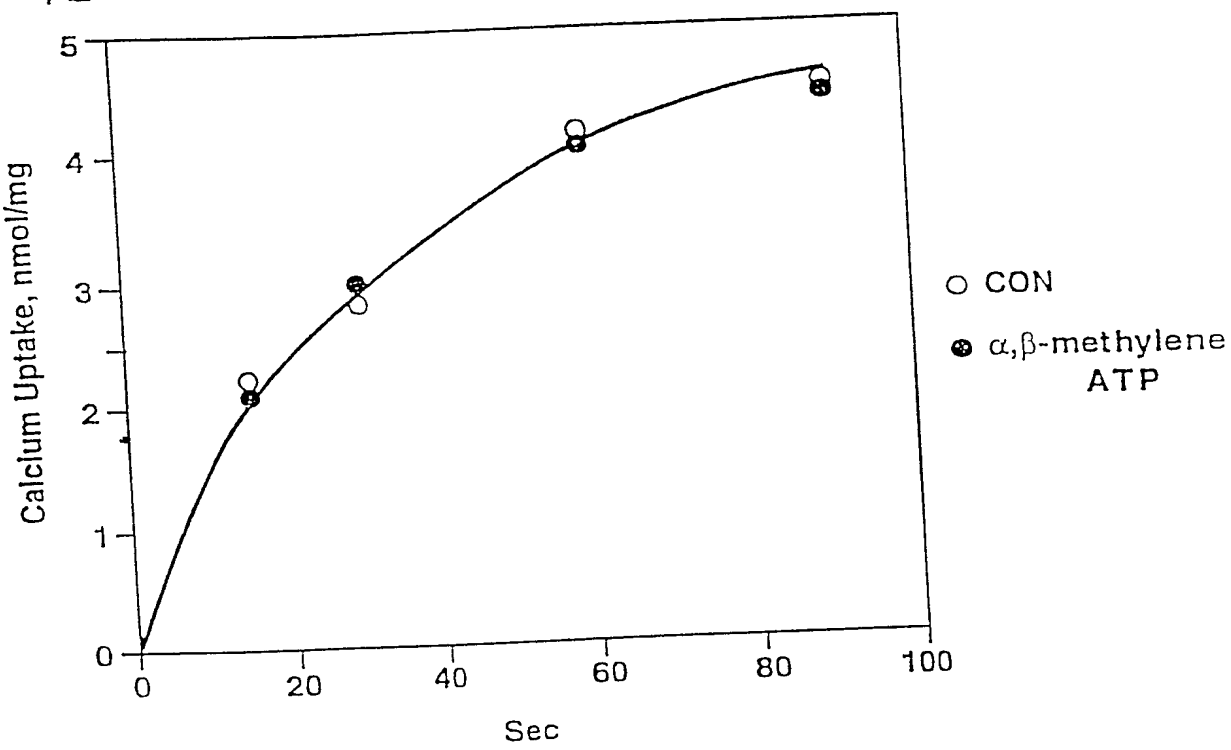

Calcium Uptake, nmol/mg
0
1
2
3
4
5
0
20
40
60
80
100
Sec
CON
α,β-methylene ATP

Fig. 8A

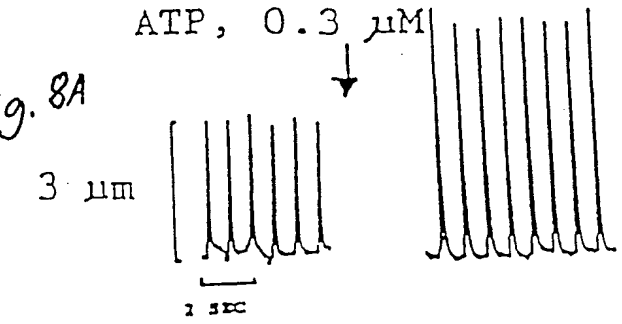


Fig. 8B

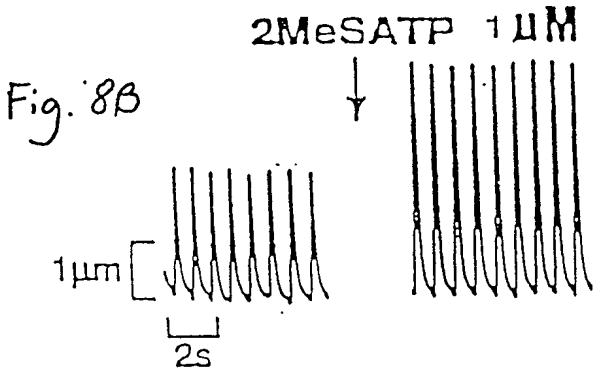


Figure 9A

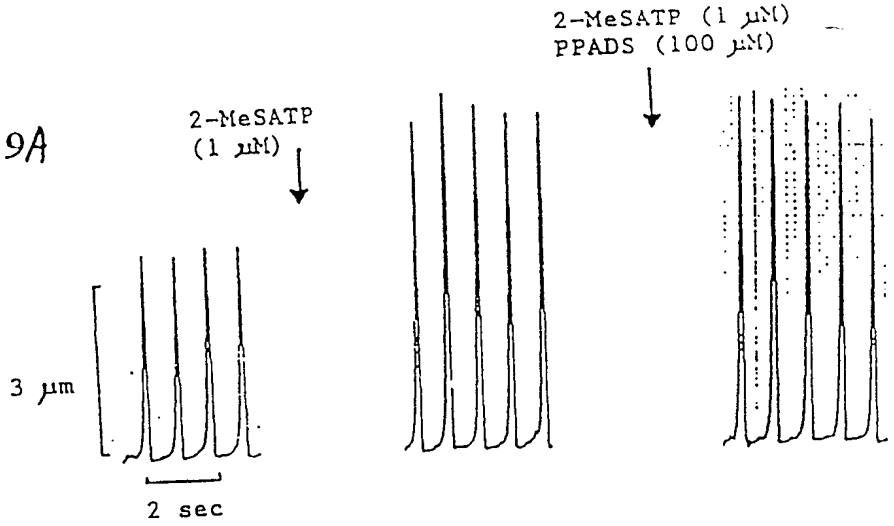
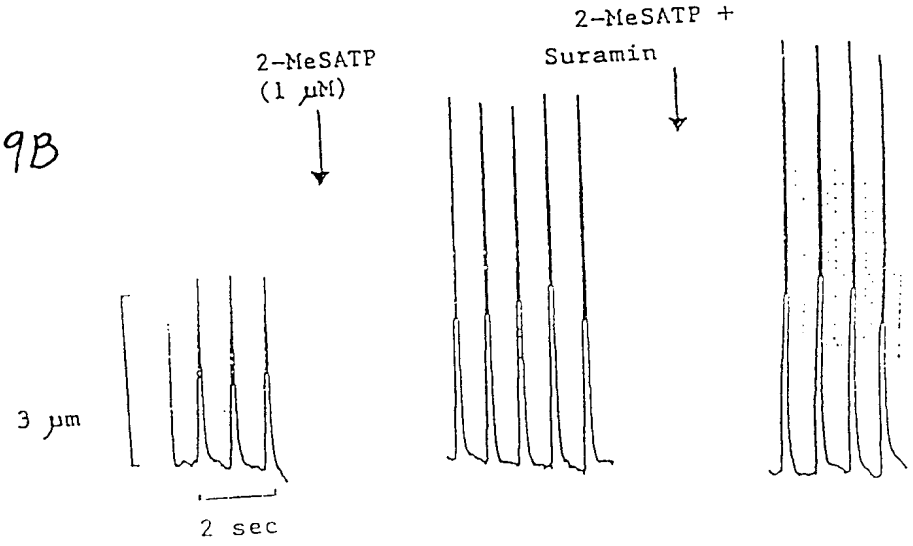


Fig. 9B



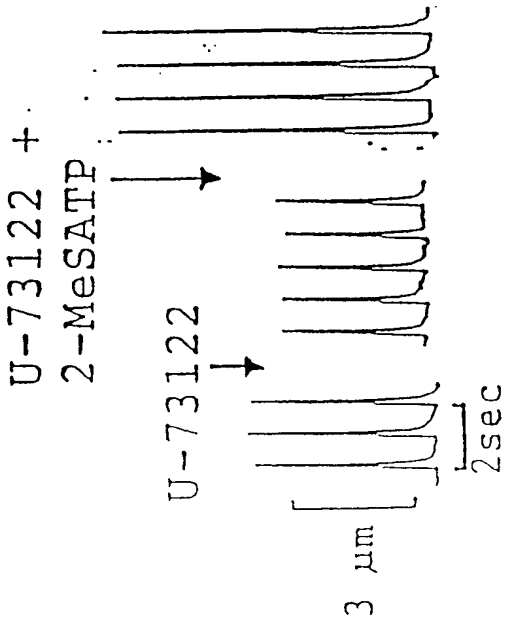


Figure 9C

Figure 10

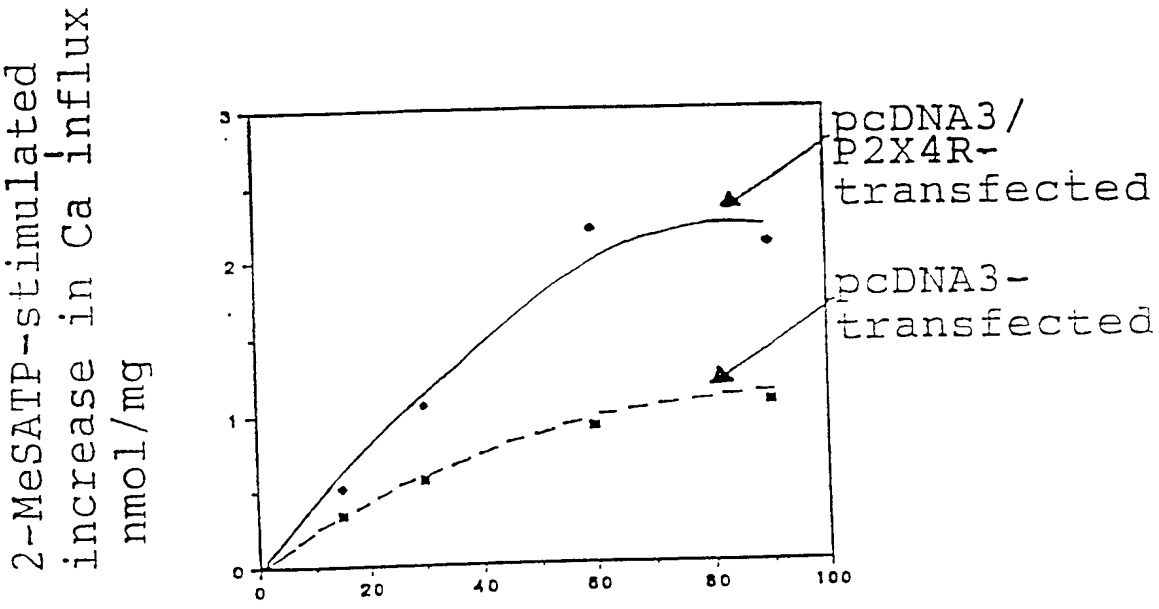

2-MeSATP-stimulated
increase in Ca influx
nmol/mg
0
1
2
3
0
20
40
60
80
100
pcDNA3/
P2X4R-
transfected
pcDNA3-
transfected

Fig. 11A

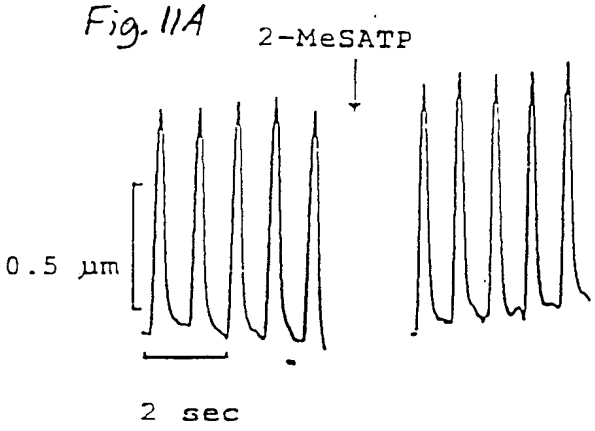


Fig. 11B

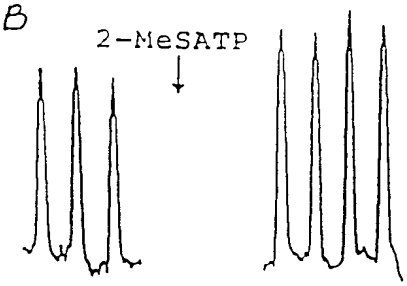


Fig. 11C

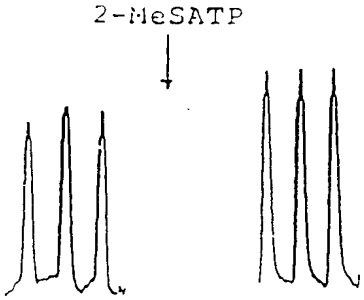


Fig. 11D

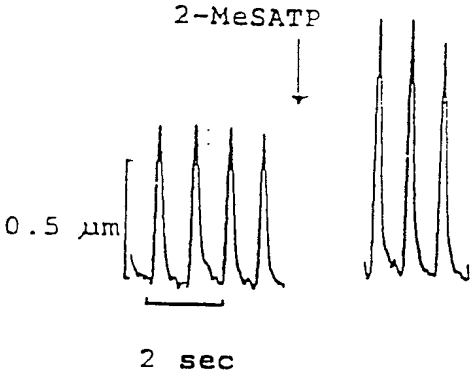


Fig. 11E

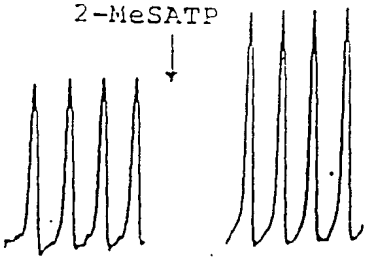
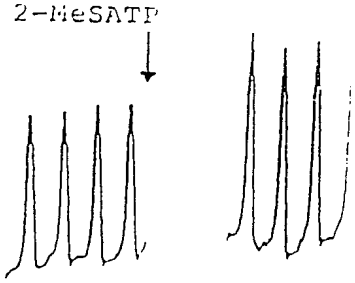


Fig. 11F



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23170

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/00, 15/12; A61K 39/00
US CL : 435/69.1, 320.1, 326; 530/350; 536/23.5; 514/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 320.1, 326; 530/350; 536/23.5; 514/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CAPLUS
search terms: myocyte, muscle, purinoreceptor, P2X, P2Y, cardiac, contractility

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MALAM-SOULEY, R. et al. Exogenous ATP induces a limited cell cycle progression of arterial smooth muscle cells. American Journal of Physiology. 1993, Vol. 264, No. 4 Part I pages C783-C788, see abstract.	1, 2, 4, 10-11, 34
Y	PACAUD, P. et al. ATP raises [Ca2+]i via different P2-receptor subtypes in freshly isolated and cultured aortic myocytes. American Journal of Physiology. 1995, Vol. 269, No. 1, Part 2, pages H30-H36, see abstract and page H35, last paragraph.	1, 2, 4, 10-11, 34

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search 15 FEBRUARY 1999	Date of mailing of the international search report 02 MAR 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ELIANE LAZAR-WESLEY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23170

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHANG, K. et al. Molecular cloning and functional analysis of a novel P2 nucleotide receptor. Journal of biological Chemistry. 03 November 1995, Vol. 270, No. 44, pages 26152-26158, especially page 26157, paragraph bridging col.1 and col.2.	1, 2, 4, 10-11, 34
Y	TOKUYAMA, Y. et al. Cloning of rat and mouse P2Y purinoreceptors. Biochemical and Biophysical Research Communications. 06 June 1995, Vol. 211, No. 1, pages 211-218, especially page 213.	1, 2, 4, 10-11, 34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23170

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-7, 10-11,33-36 as they relate to the first species, i.e. P2Y purinoreceptor.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23170

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-7, 10-11, and 33-36, drawn to a recombinant myocyte comprising at least one introduced nucleic acid encoding at least one P2 purinoreceptor, a method comprising assessing the contractile amplitude of a recombinant myocyte, and a kit. Eight species.

Group II, claims 8-9 and 12-13 , drawn to a method comprising assessing calcium uptake. Forty-eight species.

Group III, claims 14-32 , drawn to a method of enhancing cardiac contractility comprising administering to an animal a composition comprising purinoreceptors agonists and P2 purinoreceptor antagonists other than P2X4 and P2X6 antagonists, a pharmaceutical composition and a kit. Seven species.

Group IV, claim 37, drawn to a method of decreasing cardiac contractility comprising administering to an animal a purinoreceptor antagonist selected from a P2X4 and a P2X6 purinoreceptor antagonist. Two species.

The special technical feature of group I is the recombinant monocyte and the method of assessing contractility. The inventions of Groups I-IV lack unity of invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. The methods of Groups II-IV constitute different methods using different reagents and method steps and have special technical features different from the special technical features of Group I.

This application contains claims directed to more than one species of purinoreceptor used in the method of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species of purinoreceptors are as follows:
a)P2Y
b)P2X1
c)P2X2
d)P2X3
e)P2X4
f)P2X5
g)P2X6
h)P2X4/P2X6 heterodimer

The claims are deemed to correspond to the species listed above in the following manner:

a-g, claims 1-13, 33-36
a-h, claims 10-13

The following claims are generic: 1,2,4

Furthermore, claims 10-13 recite a second myocyte comprising at least one first purinoreceptor and at least one introduced nucleic acid encoding at least one second purinoreceptor.
These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:
a)P2Y
b)P2X1
c)P2X2
d)P2X3
e)P2X4
f)P2X5
g)P2X6
There appear to be forty-eight pairs of receptors.
The species listed above do not relate to a single inventive concept under PCT Rule 13.1, because under Rule PCT 13.2, the species lack the sanme or corresponding special technical features for the following reasons: they are unrelated in structure and activity.

Furthermore, this application contains claims directed to more than one species of purinoreceptor agonists and antagonists of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:
i)P2X4 purinoreceptor agonist
j)P2X6 purinoreceptor agonist
k)P2Y purinoreceptor antagonist
l)P2X1 purinoreceptor antagonist
m)P2X2 purinoreceptor antagonist
n)P2X3 purinoreceptor antagonist
o)P2X5 purinoreceptor antagonist
p)P2X4 purinoreceptor antagonist
q)P2X6 purinoreceptor antagonist

The claims are deemed to correspond to the species listed above in the following manner:

i-o, claims 14-32
p-q, claim 37

The following claims are generic: 14, 26

The species listed above do not relate to a single inventive concept under PCT Rule 13.1, because under Rule PCT 13.2, the species lack the same or corresponding special technical features for the following reasons: they are unrelated in structure and mode of action.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the reasons cited above.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the reasons cited above.

CORRECTED VERSION*

PCT

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/00, 15/12, A61K 39/00

A1

(11) International Publication Number: WO 99/23214

(43) International Publication Date: 14 May 1999 (14.05.99)

(21) International Application Number: PCT/US98/23170

(22) International Filing Date: 30 October 1998 (30.10.98)

(30) Priority Data:
60/064,329 30 October 1997 (30.10.97) US

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(74) Agents: LEARY, Kathryn, Doyle et al.; Panitch Schwarze Jacobs & Nadel, P.C., 22nd floor, One Commerce Square, 2005 Market Street, Philadelphia, PA 19103–7086 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: COMPOSITIONS AND METHODS FOR ENHANCING CARDIAC CONTRACTILITY MEDIATED BY MYOCYTE P2 PURINOCEPTORS AND MODELS THEREOF

(57) Abstract

A recombinant myocyte comprising at least one introduced nucleic acid encoding a P2 purinoceptor, such as a $P2X_4$ or $P2X_6$ purinoceptor, and a method of determining whether a compound affects cardiac contractility in an animal are provided. Furthermore, a method of augmenting cardiac contractility and a method of treating heart failure are included in the invention, as is a kit comprising one or more recombinant myocytes and an instructional material.

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COMPOSITIONS AND METHODS FOR ENHANCING CARDIAC CONTRACTILITY MEDIATED BY MYOCYTE P2 PURINOCEPTORS AND MODELS THEREOF

FIELD OF THE INVENTION

The field of the invention is cardiac myocyte contractility.

BACKGROUND OF THE INVENTION

Heart failure is the cause of significant morbidity and mortality in humans, other mammals, and other animals. Heart failure is a clinical syndrome having many different etiologies. This syndrome arises from abnormality(ies) in the mechanical performance of the heart, which reduces cardiac output to a level that is insufficient to meet the body's needs for oxygen supply and waste (e.g. CO_2) removal. Impaired cardiac function may be manifested only during exertion or, particularly in severe cases of heart failure, even when the animal is at rest. Heart failure frequently arises in conjunction with valvular, coronary, or myocardial diseases, and may furthermore be caused or aggravated by arrhythmias.

With limited exceptions, heart failure is associated with a deficiency in the contractility of cardiac myocytes, particularly in the contractility of myocytes of the mammalian (including human) left ventricle. Loss of contractility of myocytes of the mammalian right ventricle may also result in heart failure, and often arises as a consequence of persistent left ventricle failure. Clinical symptoms of heart failure are well known, and include undue tachycardia, fatigue on exertion, dyspnea on mild exercise, and intolerance to cold. Humans experiencing heart failure sometimes also exhibit pulmonary edema and associated symptoms.

Known treatments for heart failure include rest, oxygenation, correction of arrhythmias, measures to improve myocardial contractility, diuresis, and reduction of circulatory preload and afterload. Digitalis preparations, such as digoxin, digitoxin,

and quabain, are the most common agents used to enhance myocardial contractility. However, because therapeutic doses of digitalis preparations are very near toxic doses, digitalis toxicity is a common drawback to such treatment and may preclude its use in certain patients. What is needed are agents which may be used to enhance cardiac contractility without causing the toxicity associated with digitalis preparations. The present invention satisfies this need by providing such agents, kits, methods, and model systems for identifying such agents.

Cardiac Purinoceptors

Adenosine 5'-triphosphate (ATP) exerts a number of pronounced effects in the cardiovascular system (Olsson et al., 1990, Physiol. Rev. 70:761-846; Ralevic et al., 1991, Circ. 84:1-14). These effects include stimulatory effects in the heart such as vasodilatation in the coronary vasculature, stimulation of transsarcolemmal calcium entry into cardiac myocytes, acidification and depolarization of cardiac cells, cytosolic calcium transients, and a pronounced positive inotropic effect in the cardiac myocyte (Olsson et al., 1990, Physiol. Rev. 70:761-846; Ralevic et al., 1991, Circ. 84:1-14; Scamps et al., 1990, Circ. Res 67:1007-1016; Scamps et al., 1994, Br. J. Pharmacol. 113:982-986; Scamps et al., 1992, J. Gen. Physiol. 100:675-701; Puceat et al., 1991, Biochem. J. 274:55-62; Scamps et al., 1990, Pflugers Arch. 417:309-316; Danziger et al., 1988, Cell Calcium 9:193-199; DeYoung et al., 1989, Am. J. Physiol. 257(Cell Physiol. 26):C750-C758; Forrester et al., 1977, J. Physiol. (London) 268:371-390). This latter effect is mediated by a class of cell surface ATP receptors designated P2 purinoceptors (Danziger et al., 1988, Cell Calcium 9:193-199; Leggssyer et al., 1988, J. Physiol. 401:185-199).

Physiologically, ATP is released from platelets, endothelial cells, or hypoxic cardiac tissues and acts as a paracrine and autocrine regulatory agent (Borst et al., 1991, Circ. Res. 68:797-806; Clemens et al., 1980, J. Physiol. (London) 312:143-158; Forrester et al., 1977, J. Physiol. (London) 268:371-390; Fredholm et al., 1982, Acta Physiol. Scand. 116:285-295; Pearson et al., 1979, Nature 281:384-386; Vial et al., 1987, J. Mol. Cell. Cardiol. 19:187-197). Binding of ATP to P2

purinoceptors provides important inotropic support in both healthy and diseased heart tissue. When released by norepinephrine from sympathetic nerve endings, ATP is capable of acting synergistically with one or more β-adrenergic agonists to augment myocyte contractility (Zheng et al., 1992, Am. J. Physiol. 262(Cell Physiol. 31):C128-C135). The mechanism by which ATP increases myocyte contractility is not well understood.

A number of cDNA molecules have been cloned which encode P2 purinoceptors. cDNA molecules encoding at least four P2Y receptor subtypes, seven P2X receptor subtypes, and the P2 receptor found on macrophages and platelets have been cloned (Burnstock et al., 1996, Drug Develop. Res., 38:67-71).

Activation of the P2Y receptor has been shown to variably inhibit adenylyl cyclase (AC) activity and cyclic adenosine monophosphate (cAMP) accumulation, or to have no effect on AC activity and cAMP accumulation (Scamps et al., 1992, J. Gen. Physiol. 100:675-701; Yamada et al., 1992, Circ. Res. 70:477-485). P2Y receptor activation is coupled to inhibition of AC activity and cAMP accumulation via inhibitory G protein (Gi).

P2Y receptor activation is also linked to stimulation of phosphatidyl inositol 4,5-bisphosphate phospholipase C (PIP2-PLC) activity. The role of PIP2-PLC in mediating P2 receptor agonist-stimulated myocyte contractility has not been clearly understood by others. Physiologically, stimulation of PIP2-PLC activity results in intracellular accumulation of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG; Harden et al., 1995, Annu. Rev. Pharmacol. Toxicol. 35:541-579). It is possible that $InsP_3$ stimulates myocyte contractility by mobilizing intracellular calcium and that DAG increases myofilament sensitivity to calcium by activating protein kinase C (PKC), thereby enhancing cardiac contractility.

P2X receptors are a family of related ligand-gated ion channel proteins which facilitate entry of sodium and calcium ions into the cell (Brake et al., 1994, Nature 371:519-523; Chen et al., 1995, Nature 377:428-431; Valera et al., Nature

371:516-519). P2X receptor proteins are known to be present physiologically in the form of homodimers and heterodimers (e.g. a $P2X_2/P2X_3$ heterodimer).

Although it appears that P2 purinoceptors mediate stimulation of calcium entry into and accumulation within the cytosolic compartment of isolated cardiac myocytes, the subtype of P2 purinoceptor that mediates increases in cardiac myocyte contractility and the mechanism underlying this stimulatory effect have, until the present disclosure, remained unknown. Reported studies investigated the effects of ATP and ATP analogs on contractility of rat cardiac ventricular myocytes and intact papillary muscle (Danziger et al., 1988, Cell Calcium 9:193-199; Leggssyer et al., 1988, J. Physiol. 401:185-199; Scamps et al., 1990, Circ. Res 67:1007-1016). These studies clearly demonstrate that a positive inotropic response is induced by ATP. Although it was suggested that a P2Y receptor mediated the increase in the cytosolic calcium level (Bjornsson et al., 1989, Eur. J. Biochem. 186:395-404), the nature of the P2 receptor has not been determined. The cellular mechanism underlying the positive inotropic effect of ATP on cardiac myocytes remains poorly understood by others. This lack of understanding is due, at least in part, to the absence of a myocyte model for the cardiac P2 purinoceptor. Such a model would be useful for identifying compounds which affect the function of the P2 purinoceptor. The present invention provides such a model.

SUMMARY OF THE INVENTION

The invention relates to a recombinant myocyte comprising at least one introduced nucleic acid which encodes at least one P2 purinoceptor. The purinoceptor may, for example, be selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, a $P2X_4$ purinoceptor, a $P2X_5$ purinoceptor, and a $P2X_6$ purinoceptor. Preferably, the P2 purinoceptor is a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. The myocyte may, for example, be a chicken embryonic ventricular myocyte or a rat ventricular myocyte.

The invention also relates to a method of determining whether a compound enhances cardiac contractility. This method comprises assessing the contractile amplitude of a recombinant myocyte in the presence and absence of the compound. The myocyte comprises at least one first introduced nucleic acid encoding at least one of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor. An increase in the contractile amplitude of the myocyte in the presence of the compound, relative to the contractile amplitude of the myocyte in the absence of the compound, is an indication that the compound enhances cardiac contractility. Preferably, the myocyte is a chicken embryonic ventricular myocyte or a rat ventricular myocyte.

In an alternate embodiment, this method further comprises assessing the contractile amplitude of a non-recombinant myocyte in the presence and absence of the compound. If the difference between the contractile amplitude of the recombinant myocyte in the presence of the compound and the contractile amplitude of the recombinant myocyte in the absence of the compound is greater than the difference between the contractile amplitude of the non-recombinant myocyte in the presence of the compound and the contractile amplitude of the non-recombinant myocyte in the absence of the compound, then the effect of the composition on contractile amplitude is attributable to the at least one purinoceptor.

The invention further relates to a method of determining whether a compound enhances cardiac contractility. This method comprises assessing calcium uptake by a recombinant myocyte in the presence and absence of the compound. The myocyte comprises at least one first introduced nucleic acid encoding at least one of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor. An increase in calcium uptake by the myocyte in the presence of the compound, relative to calcium uptake by the myocyte in the absence of the compound, is an indication that the compound enhances cardiac contractility. In an alternate embodiment of this method calcium uptake by a non-recombinant myocyte in the presence and absence of the compound is also assessed. If the difference between calcium uptake by the recombinant myocyte in the presence of the compound and calcium uptake by the recombinant myocyte in the absence of the

compound is greater than the difference between calcium uptake by the non-recombinant myocyte in the presence of the compound and calcium uptake by the non-recombinant myocyte in the absence of the compound, then the effect of the composition on calcium uptake is attributable to the at least one purinoceptor.

The invention still further relates to a method of determining whether a compound enhances cardiac contractility. This method comprises assessing the contractile amplitude of a first myocyte and a second myocyte in the presence and absence of the compound. The first myocyte comprises at least one first P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, and a $P2X_4/P2X_6$ heterodimer. The second myocyte comprises the at least one first P2 purinoceptor and at least one introduced nucleic acid encoding at least one second P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. If the difference between the contractile amplitude of the second myocyte in the presence of the compound and the contractile amplitude of the second myocyte in the absence of the compound is greater than the difference between the contractile amplitude of the first myocyte in the presence of the compound and the contractile amplitude of the first myocyte in the absence of the compound, then the compound enhances cardiac contractility. The second P2 purinoceptor is preferably selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, and a $P2X_5$ purinoceptor.

The invention yet further relates to a method of determining whether a compound enhances cardiac contractility. This method comprises assessing calcium uptake by a first myocyte and a second myocyte in the presence and absence of the compound. The first myocyte comprises at least one first P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, and a $P2X_4/P2X_6$ heterodimer. The second myocyte comprises the at least one first P2 purinoceptor and at least one introduced nucleic acid encoding at least one second P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. If the difference between calcium uptake by the second myocyte in the presence of the compound and

calcium uptake by the second myocyte in the absence of the compound is greater than the difference between calcium uptake by the first myocyte in the presence of the compound and calcium uptake by the first myocyte in the absence of the compound, then the compound enhances cardiac contractility. In this method, the second P2 purinoceptor is preferably selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, and a $P2X_5$ purinoceptor.

The invention also relates to a method of enhancing cardiac contractility in an animal. This method comprises administering to the animal a composition comprising at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, and an antagonist of a P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. Following administration of the compound, cardiac contractility is augmented in the animal. The at least one agent is preferably selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist, for example, 2-methylthio-ATP (2-MeSATP). When 2-MeSATP is administered to the animal, it is preferably administered in an amount sufficient to effect a concentration of at least about 0.3 micromolar, and preferably at least about 3 micromolar, 2-MeSATP in a cardiac tissue of the animal. In an alternate embodiment of this method, at least one antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor is administered to the animal. This antagonist is preferably an antagonist of a P2 purinoceptor selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, and a $P2X_5$ purinoceptor, for example, suramin, reactive blue-2, or pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid. Also preferably, the at least one antagonist is administered in an amount sufficient to effect a concentration of at least about 30 micromolar, and more preferably 300 micromolar antagonist in a cardiac tissue of the animal. Preferably, the animal is a mammal, such as a human.

The invention further relates to a pharmaceutical composition for enhancing cardiac contractility. This pharmaceutical composition comprises at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, and an antagonist of a P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. Preferably, at least one agent is selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist. Also preferably, the composition comprises both a $P2X_4$ or $P2X_6$ purinoceptor agonist and at least one antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

The invention still further relates to a kit comprising a first container containing at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist and a second container containing at least one antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

The invention yet further relates to a method of treating an animal experiencing heart failure. This method comprises administering to the animal a composition comprising at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, and an antagonist of a P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. Preferably, at least one agent is selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist. Also preferably, both a $P2X_4$ or $P2X_6$ purinoceptor agonist and at least one antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor are administered to the animal.

The invention also relates to a kit for determining whether a compound enhances cardiac contractility. This kit comprises a recombinant myocyte comprising at least one introduced nucleic acid encoding at least one P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor. The kit also comprises an instructional material which describes assessment of at least one of the contractile amplitude of the myocyte and calcium uptake by the myocyte.

The invention further relates to a kit for determining whether a compound enhances cardiac contractility. This kit comprises a first myocyte and a second myocyte. The first myocyte comprises at least one first P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, and a $P2X_4/P2X_6$ heterodimer. The second myocyte comprises at least one introduced nucleic acid encoding at least one second P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

The invention still further relates to a method of determining whether a compound decreases cardiac contractility. This method comprises assessing the contractile amplitude of a recombinant myocyte in the presence and absence of the compound. The myocyte comprises at least one first introduced nucleic acid encoding at least one of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor. A decrease in the contractile amplitude of the myocyte in the presence of the compound, relative to the contractile amplitude of the myocyte in the absence of the compound, is an indication that the compound decreases cardiac contractility. In an alternate embodiment of this method, the contractile amplitude of a non-recombinant myocyte is assessed in the presence and absence of the compound. If the decrease in the contractile amplitude of the recombinant myocyte in the presence of the compound, relative to the decrease in the contractile amplitude of the recombinant myocyte in the absence of the compound, is greater than the decrease in the contractile amplitude of the non-recombinant myocyte in the presence of the compound, relative to the decrease in the contractile amplitude of the non-recombinant myocyte in the absence of the compound, then the effect of the composition on contractile amplitude is attributable to the at least one purinoceptor.

The invention also relates to a method of decreasing cardiac contractility in an animal. This method comprises administering to the animal a composition comprising at least one agent selected from the group consisting of a $P2X_4$ purinoceptor antagonist, a $P2X_6$ purinoceptor antagonist, and an agonist of a P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. Following

administration of the composition to the animal, cardiac contractility is decreased in the animal.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1, comprising Figures 1A and 1B, depicts the effects of adenine nucleotides on cultured cardiac ventricle myocyte contractile amplitude. Figure 1A is a graph which illustrates the effects of ATP, ADP, and AMP on the basal level of cardiac ventricular myocyte contractility. Figure 1B is a tracing which depicts cardiac myocyte contractility before and after treating a myocyte with 1 micromolar ATP. As illustrated in Figure 1B, the contractile amplitude was approximately 1 micrometer prior to treatment with ATP and approximately 1.8 micrometers following treatment with ATP, representing an approximately 80% increase in contractile amplitude.

Figure 2 is a graph which depicts the effects of P2 purinoceptor-selective agonists on contractile amplitude in cultured cardiac ventricular myocytes.

Figure 3, comprising Figures 3A and 3B, depicts the effects of ATP, UTP, and 2-MeSATP on intracellular inositol phosphate levels in cultured cardiac ventricular myocytes. In the graph depicted in Figure 3A, myocytes were exposed to 100 micromolar ATP (filled squares) for 30 seconds or maintained in medium not containing ATP (open squares), and the intracellular levels of $InsP_1$, InsP2, and $InsP_3$ were assessed. In the graph depicted in Figure 3B, the effects of exposing myocytes to varying concentrations of ATP, UTP, or 2-MeSATP on the intracellular level of inositol phosphates (i.e. including $InsP_1$, InsP2, and $InsP_3$) are illustrated.

Figure 4 is a bar graph which illustrates cross-desensitization of phosphoinositide hydrolysis induced by UTP and 2-MeSATP, as described herein.

Figure 5, comprising Figures 5A, 5B, 5C, and 5D, is a series of tracings which depict the effect of pretreatment with 2-MeSATP and UTP on the subsequent inotropic effect caused by addition of 2-MeSATP or UTP. The tracings depicted in Figure 5A indicate the contractile amplitude of cultured ventricular myocytes which

were pre-incubated with 100 micromolar 2-MeSATP for 18 minutes and subsequently re-challenged with 1 micromolar 2-MeSATP. The tracings depicted in Figure 5B indicate the contractile amplitude of cultured ventricular myocytes which were pre-incubated with 100 micromolar 2-MeSATP for 18 minutes and subsequently re-challenged with 10 micromolar UTP. The tracings depicted in Figure 5C indicate the contractile amplitude of cultured ventricular myocytes which were pre-incubated with 100 micromolar UTP for 19 minutes and subsequently re-challenged with 1 micromolar 2-MeSATP. The tracings depicted in Figure 5D indicate the contractile amplitude of cultured ventricular myocytes which were pre-incubated with 100 micromolar UTP for 19 minutes and subsequently re-challenged with 10 micromolar UTP.

Figure 6 is a series of tracings which depict the effect of U-73122 on the 2-MeSATP-stimulated increase in contractile amplitude of cultured cardiac ventricular myocytes.

Figure 7, comprising Figures 7A and 7B, is a pair of graphs which depict the effects of P2 purinoceptor agonists on ^{45}Ca uptake by cardiac ventricular cells. The graph depicted in Figure 7A illustrates ^{45}Ca uptake by cardiac myocytes treated with 1 micromolar 2-MeSATP (filled circles), relative to ^{45}Ca uptake by cardiac myocytes maintained in medium which did not contain 2-MeSATP (open circles). The graph depicted in Figure 7B illustrates the effects of 1 micromolar α,β-methylene-ATP on ^{45}Ca uptake by cardiac myocytes exposed to the compound (filled circles) or maintained in medium not containing the compound (open circles).

Figure 8, comprising Figures 8A and 8B is a pair of tracings which depict the effects of ATP and 2-MeSATP on the contractile amplitude of rat cardiac ventricular myocytes. The contractile amplitude of myocytes before and after addition of 0.3 micromolar ATP is illustrated in Figure 8A. The contractile amplitude of myocytes before and after addition of 1 micromolar 2-MeSATP is illustrated in Figure 8B.

Figure 9, comprising Figures 9A, 9B, and 9C is a trio of tracings which depict the effects of the P2 receptor antagonists, on the contractile amplitude of rat cardiac ventricular myocytes. The contractile amplitudes of myocytes exposed to 1 micromolar 2-MeSATP and then to 1 micromolar 2-MeSATP and 100 micromolar PPADS are illustrated in Figure 9A. The contractile amplitude of myocytes exposed to 1 micromolar 2-MeSATP and then to 1 micromolar 2-MeSATP and 100 micromolar suramin are illustrated in Figure 9B. The contractile amplitude of myocytes exposed to 10 micromolar U-73122 and then to 10 micromolar U-73122 and 1 micromolar 2-MeSATP are illustrated in Figure 9C.

Figure 10 is a graph which depicts the effect of 2-MeSATP on calcium influx of transfected cardiac ventricular myocytes. Data obtained using myocytes transfected with a vector encoding $P2X_4$ receptor are indicated by filled circles. Data obtained using myocytes transfected with a vector which did not encode $P2X_4$ receptor are indicated by filled squares.

Figure 11, comprising Figures 11A, 11B, 11C, 11D, 11E, and 11F, is a series of tracings depicting the effect of 2-MeSATP on the contractile amplitude of transfected cardiac ventricular myocytes. Data obtained using myocytes transfected with a vector encoding $P2X_4$ receptor are presented in Figures 11D, 11E, and 11F. Data obtained using myocytes transfected with a vector which did not encode $P2X_4$ receptor are presented in Figures 11A, 11B, and 11C.

DETAILED DESCRIPTION

The invention relates to the discovery that cardiac myocontractility in animals such as mammals, and particularly in humans, can be enhanced by administering to cardiac myocytes an agonist of one or both of the $P2X_4$ and $P2X_6$ purinoceptor subtypes, and that cardiac myocontractility in such animals can alternately, or further, be enhanced by administering an antagonist of a P2 purinoceptor other than the $P2X_4$ and $P2X_6$ purinoceptor subtypes to the cardiac muscle cells. Thus, known $P2X_4$ and $P2X_6$ agonist compounds, such as 2-methylthio-ATP, can be

used in place of highly toxic digitalis compositions to treat cardiac failure. Heart failure can furthermore be treated by administering to a patient experiencing, for example, arrhythmia-induced congestive heart failure a composition comprising a $P2X_4$ or $P2X_6$ agonist agent and one or more known antagonists of another P2X receptor, such as suramin, reactive blue-2, or PPADS. Alternately, one or more of the antagonists may be administered without administering an agonist.

Because only a limited number of P2 receptor agonists and antagonists are presently known, the invention further includes a recombinant myocyte model and a screening method of using such recombinant myocytes to identify compounds which enhance myocyte contractility. It will be understood by the skilled artisan that such methods can be used without adaptation to identify compounds which diminish cardiac myocyte contractility, if such compounds are desired. Such compounds include $P2X_4$ antagonists and $P2X_6$ antagonists, and are useful, for example, to treat patients afflicted with ischemic heart disease.

This screening method comprises assessing the contractile amplitude of a recombinant myocyte of the invention comprising an introduced nucleic acid encoding at least one of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor. The contractile amplitude of this recombinant myocyte is assessed in the presence and absence of a test compound. If the contractile amplitude of the recombinant myocyte in the presence of the test compound is greater than the contractile amplitude of the recombinant myocyte in the absence of the test compound, then this is an indication that the test compound enhances cardiac contractility. If the contractile amplitude of the recombinant myocyte in the presence of the test compound is less than the contractile amplitude of the recombinant myocyte in the absence of the test compound, then this is an indication that the test compound diminishes or inhibits cardiac contractility. When the myocyte is cultured on a surface, the contractile amplitude of the recombinant myocyte is preferably assessed using an opticovideo motion-detection system, as described (Barry et al., 1982, J. Physiol. 325:243-260). However, it is understood that any method of assessing muscle cell contractility may be used.

Alternately, according to this screening method, the change in the contractile amplitude of the recombinant myocyte attributable to the presence of the test compound may be compared with the change in the contractile amplitude of a non-recombinant myocyte (i.e. a myocyte which does not comprise the introduced nucleic acid) attributable to the presence of the test compound. If the contractile amplitude of the recombinant myocyte in the presence of the test compound is greater than the contractile amplitude of the non-recombinant myocyte in the presence of the test compound, then this is an indication that the test compound enhances cardiac contractility. If the difference between the contractile amplitude of the recombinant myocyte in the presence of the test compound and the contractile amplitude of the recombinant myocyte in the absence of the test compound, is greater than the difference between the contractile amplitude of the non-recombinant myocyte in the presence of the test compound and the contractile amplitude of the non-recombinant myocyte in the absence of the test compound, then this is also an indication that the test compound enhances cardiac contractility.

The contractile amplitude of the myocyte may be assessed using any method known in the art including, but not limited, the methods described herein. Thus, the contractile amplitude may be assessed by microscopically examining the myocyte, by using an opticovideo motion-detection system, and the like.

Calcium uptake by myocytes may be assessed instead of contractile amplitude, it being understood that increased calcium uptake is associated with increased contractility in myocytes such as cardiac myocytes. Therefore, assessment of calcium uptake may be performed in place of assessment of contractile amplitude wherever assessment of contractile amplitude is mentioned in the present disclosure.

In these assay methods, the myocyte may comprise an introduced nucleic acid encoding one purinoceptor, an introduced nucleic acid encoding a plurality of purinoceptors, or a plurality of introduced nucleic acids, each encoding the same or different purinoceptors, as described elsewhere herein.

When myocytes comprising an introduced nucleic acid encoding one purinoceptor are used, it is possible to assess the effect of test compounds on contractile amplitude modifications attributable to that receptor. Thus, for example, using a first recombinant myocyte comprising an introduced nucleic acid encoding a $P2X_4$ purinoceptor and a second recombinant myocyte comprising an introduced nucleic acid encoding both a $P2X_4$ purinoceptor and a $P2X_5$ purinoceptor, it is possible to identify compounds which are not $P2X_4$ agonists, but which, in the presence of a $P2X_4$ agonist, further enhance cardiac myocyte contractility by virtue of being $P2X_5$ antagonists.

The screening method may be performed using a first myocyte comprising a $P2X_4$ or $P2X_6$ purinoceptor and a second (recombinant) myocyte comprising the same $P2X_4$ or $P2X_6$ purinoceptor and an introduced nucleic acid encoding one or more P2 purinoceptors other than the $P2X_4$ or $P2X_6$ purinoceptor. The first myocyte may be recombinant or not. The screening method is performed by assessing contractility of the first myocyte in the presence of an agonist of the $P2X_4$ or $P2X_6$ purinoceptor and in the presence and absence of a test compound and contractility of the second myocyte in the presence of the agonist and in the presence and absence of the test compound. If contractility is increased in the presence of the test compound in the second myocyte more than in the first myocyte, then the test compound is an antagonist of at least one (non-$P2X_4$ or -$P2X_6$) P2 purinoceptor encoded by the introduced nucleic acid of the second myocyte.

The invention also includes a recombinant myocyte comprising at least one introduced nucleic acid encoding at least one P2 purinoceptor. The introduced nucleic acid is constructed such that it is expressed in the myocyte. Innumerable nucleic acid expression constructs are known in the art, and the introduced nucleic acid(s) may comprise essentially any such construct. The P2 purinoceptor encoded by the recombinant gene may be a purinoceptor encoded by a nucleic acid normally contained within a cell of the animal or may be a purinoceptor encoded by a nucleic acid obtained from a different animal, such as a human. In either case, the myocyte

comprising the introduced nucleic acid expresses this P2 purinoceptor at a higher level than the same myocyte not comprising the introduced nucleic acid. The myocyte may comprise one or more introduced nucleic acid, each encoding the same or different P2 purinoceptors.

The myocyte may be one obtained from any animal or may be cultured from an embryo of any animal. The animal is preferably a mammal, such as a rat or a human, or an animal from which myocytes may be easily obtained, such as a chick. The myocyte is preferably a cardiac myocyte such as an adult rat ventricular myocyte or a ventricular myocyte obtained by culturing a ventricular cell obtained from a chick embryo as described herein.

The P2 purinoceptor may be any P2 purinoceptor, and is preferably a P2X purinoceptor or a P2Y purinoceptor. Contemplated P2X receptors include, for example, a $P2X_1$ receptor, a $P2X_2$ receptor, a $P2X_3$ receptor, a $P2X_4$ receptor, a $P2X_5$ receptor, and a $P2X_6$ receptor. Preferably, the P2X purinoceptor is a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, or a $P2X_4/P2X_6$ heterodimer. The P2 purinoceptor may be a normal component of the myocyte which is expressed at a higher level when the myocyte comprises the introduced nucleic acid, or the P2 purinoceptor may be one which is expressed by the myocyte only when the myocyte comprises the introduced nucleic acid. A $P2X_4/P2X_6$ heterodimer may be expressed by a myocyte when the cell expresses both $P2X_4$ and $P2X_6$ purinoceptors. Preferably, the purinoceptor is a mammalian purinoceptor, more preferably a human purinoceptor.

The recombinant myocyte may be made by preparing a nucleic acid vector comprising the introduced nucleic acid and delivering the nucleic acid vector to an animal myocyte. Any known method of preparing a nucleic acid vector and any known method of transforming or transfecting an animal cell using the vector may be used. By way of example, the vector may be the plasmid designated pcDNA3, the recombinant gene may be incorporated into the plasmid using standard molecular biology techniques, and a chick embryonic ventricular myocyte may be transfected

using the plasmid by performing the modified calcium phosphate transfection method described by Xu et al., (1992, Nucl. Acids Res. 20:6425-6426).

The invention also includes a kit for determining whether a compound enhances cardiac contractility. In one embodiment, the kit comprises a recombinant myocyte comprising an introduced nucleic acid which encodes a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, or both. The kit further comprises an instructional material, as described herein, which describes assessing the contractile amplitude of the myocyte or assessing calcium uptake by the myocyte. By culturing the myocyte in the presence and absence of the compound and assessing the contractile amplitude of the myocyte, or assessing calcium uptake by the myocyte, one may determine whether the compound is an agonist of the purinoceptor(s), an antagonist of the purinoceptor(s), or neither.

In another embodiment of the kit of the invention, the kit comprises a first myocyte comprising a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, or both, and a second recombinant myocyte comprising the $P2X_4$ purinoceptor, the $P2X_6$ purinoceptor, or both, and further comprising at least one introduced nucleic acid encoding at least one second P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor. The first myocyte may be recombinant or not, and the second myocyte is preferably of the same type as the first. The kit may further include an instructional material which describes assessment of the contractile amplitude of the myocytes. By comparing the change in the contractile amplitude of the first myocyte upon exposure to a test compound with the change in the contractile amplitude of the second myocyte upon exposure to the test compound, one may determine whether the test compound is an agonist, an antagonist, or neither, of the second P2 purinoceptor(s). If the test compound is an agonist of the second P2 purinoceptor(s), then the increase in the contractile amplitude of the second myocyte upon exposure to the test compound will be greater than the increase in the contractile amplitude of the first myocyte upon exposure to the test compound. If the test compound is an antagonist of the second P2 purinoceptor(s), then the increase in the contractile amplitude of the second myocyte

upon exposure to the test compound will be less than the increase in the contractile amplitude of the first myocyte upon exposure to the test compound.

Agonists of $P2X_4$ and $P2X_6$ purinoceptors are useful for treatment of any diseases associated with sub-normal myocyte contractility, such as heart failure. Likewise, antagonists of P2 purinoceptors other than $P2X_4$ and $P2X_6$ purinoceptors are useful for treatment of any diseases associated with sub-normal myocyte contractility. Such diseases include, but are not limited to heart attack or myocardial infarction, myocarditis, and various cardiomyopathies. Thus, given the disclosure provided herein, it is possible to augment cardiac contractility in an animal by administering to the animal a composition comprising a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, an antagonist of a purinoceptor other than $P2X_4$ and $P2X_6$, or some combination of these agonists and antagonists. Such a composition may, of course, further comprise a pharmaceutically acceptable carrier, particularly where the composition is intended for administration to humans.

The invention encompasses the preparation and use of medicaments or pharmaceutical compositions comprising a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, an antagonist of a purinoceptor other than $P2X_4$ and $P2X_6$, or some combination of these agonists and antagonists as an active ingredient(s). Such a pharmaceutical composition may consist of the active ingredient(s) alone, in a form suitable for administration to a subject, or the pharmaceutical composition may comprise the active ingredient(s) and one or more pharmaceutically acceptable carriers, one or more additional ingredients, or some combination of these. Administration of one of these pharmaceutical compositions to a subject is useful for enhancing cardiac contractility in the subject, as described elsewhere in the present disclosure. The active ingredient(s) may be present in the pharmaceutical composition in the form of a physiologically acceptable ester or salt, such as in combination with a physiologically acceptable cation or anion, as is well known in the art.

As used herein, the term "pharmaceutically acceptable carrier" means a chemical composition with which the active ingredient(s) may be combined and which,

following the combination, can be used to administer the active ingredient(s) to a subject, and which is not deleterious to the subject.

As used herein, the term "physiologically acceptable" ester or salt means an ester or salt form of the active ingredient(s) which is compatible with any other ingredients of the pharmaceutical composition and which is not deleterious to the subject to which the composition is to be administered.

The formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient(s) into association with a carrier or one or more other accessory ingredients, and then, if necessary or desirable, shaping or packaging the product into a desired single- or multi-dose unit.

Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for ethical administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to animals of all sorts. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions of the invention is contemplated include, but are not limited to, humans and other primates, mammals including commercially relevant mammals such as cattle, pigs, horses, sheep, cats, and dogs, birds including commercially relevant birds such as chickens, ducks, geese, and turkeys, fish including farm-raised fish and aquarium fish, and crustaceans such as farm-raised shellfish.

Pharmaceutical compositions that are useful in the methods of the invention may be prepared, packaged, or sold in formulations suitable for oral, rectal, vaginal, parenteral, topical, pulmonary, intranasal, buccal, ophthalmic, or another route

of administration which can be used to deliver a P2 purinoceptor agonist or antagonist of the invention to a cardiac myocardial tissue. Other contemplated formulations include projected nanoparticles, liposomal preparations, resealed erythrocytes containing the active ingredient(s), and immunologically-based formulations.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient(s). The amount of the active ingredient is generally equal to the dosage of the active ingredient(s) which would be administered to a subject or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

The relative amounts of the active ingredient(s), the pharmaceutically acceptable carrier, and any additional ingredients in a pharmaceutical composition of the invention will vary, depending upon the identity, size, and condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100% (w/w) active ingredient(s).

In addition to the active ingredient(s), a pharmaceutical composition of the invention may further comprise one or more additional pharmaceutically active agents.

Controlled- or sustained-release formulations of a pharmaceutical composition of the invention may be made using conventional technology.

A formulation of a pharmaceutical composition of the invention suitable for oral administration may be prepared, packaged, or sold in the form of a discrete solid dose unit including, but not limited to, a tablet, a hard or soft capsule, a cachet, a troche, or a lozenge, each containing a predetermined amount of the active ingredient(s). Other formulations suitable for oral administration include, but are not limited to, a powdered or granular formulation, an aqueous or oily suspension, an aqueous or oily solution, or an emulsion.

As used herein, an "oily" liquid is one which comprises a carbon-containing liquid molecule and which exhibits a less polar character than water.

A tablet comprising the active ingredient(s) may, for example, be made by compressing or molding the active ingredient(s), optionally with one or more additional ingredients. Compressed tablets may be prepared by compressing, in a suitable device, the active ingredient(s) in a free-flowing form such as a powder or granular preparation, optionally mixed with one or more of a binder, a lubricant, an excipient, a surface active agent, and a dispersing agent. Molded tablets may be made by molding, in a suitable device, a mixture of the active ingredient(s), a pharmaceutically acceptable carrier, and at least sufficient liquid to moisten the mixture. Pharmaceutically acceptable excipients used in the manufacture of tablets include, but are not limited to, inert diluents, granulating and disintegrating agents, binding agents, and lubricating agents. Known dispersing agents include, but are not limited to, potato starch and sodium starch glycolate. Known surface active agents include, but are not limited to, sodium lauryl sulphate. Known diluents include, but are not limited to, calcium carbonate, sodium carbonate, lactose, microcrystalline cellulose, calcium phosphate, calcium hydrogen phosphate, and sodium phosphate. Known granulating and disintegrating agents include, but are not limited to, corn starch and alginic acid. Known binding agents include, but are not limited to, gelatin, acacia, pre-gelatinized maize starch, polyvinylpyrrolidone, and hydroxypropyl methylcellulose. Known lubricating agents include, but are not limited to, magnesium stearate, stearic acid, silica, and talc.

Tablets may be non-coated or they may be coated using known methods to achieve delayed disintegration in the gastrointestinal tract of a subject, thereby providing sustained release and absorption of the active ingredient(s). By way of example, a material such as glyceryl monostearate or glyceryl distearate may be used to coat tablets. Further by way of example, tablets may be coated using methods described in U.S. Patents numbers 4,256,108; 4,160,452; and 4,265,874 to form osmotically-controlled release tablets. Tablets may further comprise a sweetening

agent, a flavoring agent, a coloring agent, a preservative, or some combination of these in order to provide pharmaceutically elegant and palatable preparation.

Hard capsules comprising the active ingredient(s) may be made using a physiologically degradable composition, such as gelatin. Such hard capsules comprise the active ingredient(s), and may further comprise additional ingredients including, for example, an inert solid diluent such as calcium carbonate, calcium phosphate, or kaolin.

Soft gelatin capsules comprising the active ingredient(s) may be made using a physiologically degradable composition, such as gelatin. Such soft capsules comprise the active ingredient(s), which may be mixed with water or an oil medium such as peanut oil, liquid paraffin, or olive oil.

Liquid formulations of a pharmaceutical composition of the invention which are suitable for oral administration may be prepared, packaged, and sold either in liquid form or in the form of a dry product intended for reconstitution with water or another suitable vehicle prior to use.

Liquid suspensions may be prepared using conventional methods to achieve suspension of the active ingredient(s) in an aqueous or oily vehicle. Aqueous vehicles include, for example, water and isotonic saline. Oily vehicles include, for example, almond oil, oily esters, ethyl alcohol, vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin. Liquid suspensions may further comprise one or more additional ingredients including, but not limited to, suspending agents, dispersing or wetting agents, emulsifying agents, demulcents, preservatives, buffers, salts, flavorings, coloring agents, and sweetening agents. Oily suspensions may further comprise a thickening agent. Known suspending agents include, but are not limited to, sorbitol syrup, hydrogenated edible fats, sodium alginate, polyvinylpyrrolidone, gum tragacanth, gum acacia, and cellulose derivatives such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose. Known dispersing or wetting agents include, but are not limited to, naturally-occurring phosphatides such as lecithin,

condensation products of an alkylene oxide with a fatty acid, with a long chain aliphatic alcohol, with a partial ester derived from a fatty acid and a hexitol, or with a partial ester derived from a fatty acid and a hexitol anhydride (e.g. polyoxyethylene stearate, heptadecaethyleneoxycetanol, polyoxyethylene sorbitol monooleate, and polyoxyethylene sorbitan monooleate, respectively). Known emulsifying agents include, but are not limited to, lecithin and acacia. Known preservatives include, but are not limited to, methyl, ethyl, or n-propyl-para- hydroxybenzoates, ascorbic acid, and sorbic acid. Known sweetening agents include, for example, glycerol, propylene glycol, sorbitol, sucrose, and saccharin. Known thickening agents for oily suspensions include, for example, beeswax, hard paraffin, and cetyl alcohol.

Liquid solutions of the active ingredient(s) in aqueous or oily solvents may be prepared in substantially the same manner as liquid suspensions, the primary difference being that the active ingredient(s) is dissolved, rather than suspended in the solvent. Liquid solutions of the pharmaceutical composition of the invention may comprise each of the components described with regard to liquid suspensions, it being understood that suspending agents will not necessarily aid dissolution of the active ingredient(s) in the solvent. Aqueous solvents include, for example, water and isotonic saline. Oily solvents include, for example, almond oil, oily esters, ethyl alcohol, vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin.

Powdered and granular formulations of a pharmaceutical preparation of the invention may be prepared using known methods. Such formulations may be administered directly to a subject, used, for example, to form tablets, to fill capsules, or to prepare an aqueous or oily suspension or solution by addition of an aqueous or oily vehicle thereto. Each of these formulations may further comprise one or more of dispersing or wetting agent, a suspending agent, and a preservative. Additional excipients, such as fillers and sweetening, flavoring, or coloring agents, may also be included in these formulations.

A pharmaceutical composition of the invention may also be prepared, packaged, or sold in the form of oil-in-water emulsion or a water-in-oil emulsion. The oily phase may be a vegetable oil such as olive or arachis oil, a mineral oil such as liquid paraffin, or a combination of these. Such compositions may further comprise one or more emulsifying agents such as naturally occurring gums such as gum acacia or gum tragacanth, naturally-occurring phosphatides such as soybean or lecithin phosphatide, esters or partial esters derived from combinations of fatty acids and hexitol anhydrides such as sorbitan monooleate, and condensation products of such partial esters with ethylene oxide such as polyoxyethylene sorbitan monooleate. These emulsions may also contain additional ingredients including, for example, sweetening or flavoring agents.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for rectal administration. Such a composition may be in the form of, for example, a suppository, a retention enema preparation, and a solution for rectal or colonic irrigation.

Suppository formulations may be made by combining the active ingredient(s) with a non-irritating pharmaceutically acceptable excipient which is solid at ordinary room temperature (i.e. about 20°C) and which is liquid at the rectal temperature of the subject (i.e. about 37°C in a healthy human). Suitable pharmaceutically acceptable excipients include, but are not limited to, cocoa butter, polyethylene glycols, and various glycerides. Suppository formulations may further comprise various additional ingredients including, but not limited to, antioxidants and preservatives.

Retention enema preparations or solutions for rectal or colonic irrigation may be made by combining the active ingredient(s) with a pharmaceutically acceptable liquid carrier. As is well known in the art, enema preparations may be administered using, and may be packaged within, a delivery device adapted to the rectal anatomy of the subject. Enema preparations may further comprise various additional ingredients including, but not limited to, antioxidants and preservatives.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for vaginal administration. Such a composition may be in the form of, for example, a suppository, an impregnated or coated vaginally-insertable material such as a tampon, a douche preparation, or a solution for vaginal irrigation.

Methods for impregnating or coating a material with a chemical composition are known in the art, and include, but are not limited to methods of depositing or binding a chemical composition onto a surface, methods of incorporating a chemical composition into the structure of a material during the synthesis of the material (i.e. such as with a physiologically degradable material), and methods of absorbing an aqueous or oily solution or suspension into an absorbent material, with or without subsequent drying.

Douche preparations or solutions for vaginal irrigation may be made by combining the active ingredient(s) with a pharmaceutically acceptable liquid carrier. As is well known in the art, douche preparations may be administered using, and may be packaged within, a delivery device adapted to the vaginal anatomy of the subject. Douche preparations may further comprise various additional ingredients including, but not limited to, antioxidants, antibiotics, antifungal agents, and preservatives.

As used herein, "parenteral administration" of a pharmaceutical composition includes any route of administration characterized by physical breaching of a tissue of a subject and administration of the pharmaceutical composition through the breach in the tissue. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, subcutaneous, intraperitoneal, intravenous, intraarterial, intramuscular, or intrasternal injection and intravenous, intraarterial, or kidney dialytic infusion techniques.

Formulations of a pharmaceutical composition suitable for parenteral administration comprise the active ingredient(s) combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules or in multi-dose containers containing a preservative. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for parenteral administration, the active ingredient(s) is provided in dry (i.e. powder or granular) form for reconstitution with a suitable vehicle (e.g. sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition.

The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient(s), additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations may be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations which are useful include those which comprise the active ingredient(s) in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer systems. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

Formulations suitable for topical administration include, but are not limited to, liquid or semi-liquid preparations such as liniments, lotions, oil-in-water or water-in-oil emulsions such as creams, ointments or pastes, and solutions or suspensions. Topically-administrable formulations may, for example, comprise from about 1% to about 10% (w/w) active ingredient(s), although the concentration of the active ingredient(s) may be as high as the solubility limit of the active ingredient(s) in the solvent. Formulations for topical administration may further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for pulmonary administration via the buccal cavity. Such a formulation may comprise dry particles which comprise the active ingredient(s) and which have a diameter in the range from about 0.5 to about 7 nanometers, and preferably from about 1 to about 6 nanometers. Such compositions are conveniently in the form of dry powders for administration using a device comprising a dry powder reservoir to which a stream of propellant may be directed to disperse the powder or using a self-propelling solvent/powder-dispensing container such as a device comprising the active ingredient(s) dissolved or suspended in a low-boiling propellant in a sealed container. Preferably, such powders comprise particles wherein at least 98% of the particles by weight have a diameter greater than 0.5 nanometers and at least 95% of the particles by number have a diameter less than 7 nanometers. More preferably, at least 95% of the particles by weight have a diameter greater than 1 nanometer and at least 90% of the particles by number have a diameter less than 6 nanometers. Dry powder compositions preferably include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form.

Low boiling propellants generally include liquid propellants having a boiling point of below 65°F at atmospheric pressure. Generally the propellant may constitute 50 to 99.9% (w/w) of the composition, and the active ingredient(s) may constitute 0.1 to 20% (w/w) of the composition. The propellant may further comprise additional ingredients such as a liquid non-ionic or solid anionic surfactant or a solid

diluent (preferably having a particle size of the same order as particles comprising the active ingredient(s)).

Pharmaceutical compositions of the invention formulated for pulmonary delivery may also provide the active ingredient(s) in the form of droplets of a solution or suspension. Such formulations may be prepared, packaged, or sold as aqueous or dilute alcoholic solutions or suspensions, optionally sterile, comprising the active ingredient(s), and may conveniently be administered using any nebulization or atomization device. Such formulations may further comprise one or more additional ingredients including, but not limited to, a flavoring agent such as saccharin sodium, a volatile oil, a buffering agent, a surface active agent, or a preservative such as methylhydroxybenzoate. The droplets provided by this route of administration preferably have an average diameter in the range from about 0.1 to about 200 nanometers.

The formulations described herein as being useful for pulmonary delivery are also useful for intranasal delivery of a pharmaceutical composition of the invention.

Another formulation suitable for intranasal administration is a coarse powder comprising the active ingredient(s) and having an average particle from about 0.2 to 500 micrometers. Such a formulation is administered in the manner in which snuff is taken i.e. by rapid inhalation through the nasal passage from a container of the powder held close to the nares.

Formulations suitable for nasal administration may, for example, comprise from about as little as 0.1% (w/w) and as much as 100% (w/w) of the active ingredient(s), and may further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for buccal administration. Such formulations may, for example, be in the form of tablets or lozenges made using conventional methods, and may, for example, 0.1 to 20% (w/w) active ingredient(s),

the balance comprising an orally dissolvable or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for buccal administration may comprise a powder or an aerosolized or atomized solution or suspension comprising the active ingredient(s). Such powdered, aerosolized, or aerosolized formulations, when dispersed, preferably have an average particle or droplet size in the range from about 0.1 to about 200 nanometers, and may further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for ophthalmic administration. Such formulations may, for example, be in the form of eye drops including, for example, a 0.1-1.0% (w/w) solution or suspension of the active ingredient(s) in an aqueous or oily liquid carrier. Such drops may further comprise buffering agents, salts, or one or more other of the additional ingredients described herein. Other ophthalmalmically-administrable formulations which are useful include those which comprise the active ingredient(s) in microcrystalline form or in a liposomal preparation.

As used herein, "additional ingredients" include, but are not limited to, one or more of the following: excipients; surface active agents; dispersing agents; inert diluents; granulating and disintegrating agents; binding agents; lubricating agents; sweetening agents; flavoring agents; coloring agents; preservatives; physiologically degradable compositions such as gelatin; aqueous vehicles and solvents; oily vehicles and solvents; suspending agents; dispersing or wetting agents; emulsifying agents, demulcents; buffers; salts; thickening agents; fillers; emulsifying agents; antioxidants; antibiotics; antifungal agents; stabilizing agents; and pharmaceutically acceptable polymeric or hydrophobic materials. Other "additional ingredients" which may be included in the pharmaceutical compositions of the invention are known in the art and described, for example in Genaro, ed., 1985, Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, which is incorporated herein by reference.

A pharmaceutical composition of the invention may be administered to deliver a dose of between 1 ng/kg/day and 100 mg/kg/day, and preferably to deliver an amount of a $P2X_4$ or $P2X_6$ agonist sufficient to effect in a cardiac tissue of the subject an agonist concentration from at least about the EC_{50} value *in vitro* to ten times, or even one hundred times, that value. Alternately, or in addition, the pharmaceutical composition of the invention may be administered to deliver a dose of between 1 ng/kg/day and 100 mg/kg/day, and preferably to deliver an amount of an antagonist of a P2 receptor other than $P2X_4$ or $P2X_6$ sufficient to effect in a cardiac tissue of the subject an antagonist concentration from at least about the IC_{50} value *in vitro* to ten times, or even one hundred times, that value. The EC_{50} value of a $P2X_4$ or $P2X_6$ agonist is the concentration of the agonist which effects a half-maximal change in the contractility of a myocyte, and may be readily determined by the ordinarily skilled artisan in view of the present disclosure. Similarly, the IC_{50} value of an antagonist of a P2 receptor other than $P2X_4$ or $P2X_6$ is the concentration of the antagonist which effects a half-maximal change in the contractility of a myocyte comprising a $P2X_4$, $P2X_6$, or both, receptors, and may be readily determined by the ordinarily skilled artisan in view of the present disclosure. When the $P2X_4$ or $P2X_6$ agonist is 2-methylthio-ATP, the agonist is preferably administered in an amount sufficient to effect a concentration of at least about 0.3 micromolar, and more preferably at least about 3 micromolar, 2-methylthio-ATP in a cardiac tissue (e.g. left or right ventricle muscle tissue) of the subject. When the antagonist of a P2 receptor other than $P2X_4$ or $P2X_6$ is suramin, reactive blue-2, or PPADS, the antagonist is preferably administered in an amount sufficient to effect a concentration of at least about 30 micromolar, and more preferably at least about 300 micromolar, antagonist in a cardiac tissue of the subject

It is understood that the ordinarily skilled physician or veterinarian will readily determine and prescribe an effective amount of the compound to enhance cardiac contractility in the subject. In so proceeding, the physician or veterinarian may, for example, prescribe a relatively low dose at first, subsequently increasing the dose

until an appropriate response is obtained. It is further understood, however, that the specific dose level for any particular subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the severity of the myocardial condition (e.g. heart failure) being treated. Furthermore, determination of an appropriate amount of an agonist or antagonist to administer to a subject by way of any particular route of administration in order to achieve the effective amounts of the agonists and antagonists described herein involves application of standard pharmacological principles and experience which may be performed by the ordinarily skilled artisan in view of the present disclosure.

Another aspect of the invention relates to a kit comprising one or more pharmaceutical compositions of the invention. In one aspect, the kit comprises a first pharmaceutical composition comprising an agonist of a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, or both, and a second pharmaceutical composition comprising at least one antagonist of at least one purinoceptor other than a $P2X_4$ or $P2X_6$ purinoceptor. For example, such a kit may comprise separate vials of the two pharmaceutical compositions, or it may further comprise an instructional material which describes administration of one, the other, or both, pharmaceutical compositions to an animal, such as a human, who is experiencing heart failure.

As used herein, an "instructional material" includes a publication, a recording, a diagram, or any other medium of expression which is used to communicate, for example, the usefulness of the pharmaceutical composition of the invention for enhancing cardiac contractility in a subject. The instructional material may also, for example, describe an appropriate dose of the pharmaceutical composition of the invention. The instructional material of the kit of the invention may, for example, be affixed to a container which contains a pharmaceutical composition of the invention or be shipped together with a container which contains the pharmaceutical composition. Alternatively, the instructional material may be shipped separately from

the container with the intention that the instructional material and the pharmaceutical composition be used cooperatively by the recipient.

The kit may further comprise a delivery device for delivering a pharmaceutical composition of the invention to a subject. By way of example, the delivery device may be a squeezable spray bottle, a metered-dose spray bottle, an aerosol spray device, an atomizer, a dry powder delivery device, a self-propelling solvent/powder-dispensing device, a syringe, a needle, a tampon, or a dosage measuring container. The kit may further comprise an instructional material as described herein.

Given the disclosure provided herein, it is possible to treat an animal such as a mammal (e.g. a human) experiencing heart failure by administering to the animal a pharmaceutical composition of the invention. It will be understood that such pharmaceutical compositions may be used to enhance cardiac contractility in an animal for any reason.

Definitions

As used herein, an "introduced" nucleic acid is a nucleic acid which does not naturally occur in a cell. Examples of introduced nucleic acids include nucleic acid vectors such as plasmids, virus vectors, naked DNA and the like.

As used herein, a "recombinant" myocyte is one which comprises a nucleic acid which is not present in a naturally occurring form in the myocyte (i.e. an "introduced" nucleic acid) and which encodes at least one P2 purinoceptor. The introduced nucleic acid may, for example, be a nucleic acid encoding a protein which is naturally expressed by a species different than the species from which the myocyte was obtained. Further by way of example, the introduced nucleic acid may comprise an additional copy of a nucleic acid which naturally occurs in the myocyte (i.e. yielding a myocyte with a non-naturally occurring number of copies of the nucleic acid).

The term "non-recombinant" myocyte is given a special meaning herein, and means a myocyte which does not comprise an introduced nucleic acid which encodes a P2 purinoceptor. Non-recombinant myocytes may thus comprise one or

more introduced nucleic acids (e.g. a nucleic acid vector used for transformation of cells) which do not encode a P2 purinoceptor.

As used herein, the "contractile amplitude" of a myocyte means the geometric magnitude of contractile motion exhibited by a contractile cell such as a myocyte.

A compound "enhances" cardiac contractility if the contractile amplitude of cardiac myocytes is greater in the presence of the compound than in the absence of the compound.

As used herein, an "agonist" of a P2 purinoceptor is a compound which activates the purinoceptor.

As used herein, an "antagonist" of a P2 purinoceptor is a compound which blocks or inhibits activation of the purinoceptor by an agonist of the purinoceptor.

The invention is now described with reference to the following Experimental Example. This Experimental Example is provided for the purpose of illustration only and the invention should in no way be construed as being limited to this Experimental Example, but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Experimental Example

The invention is now described with reference to the following Experimental Example. This Example is provided for the purpose of illustration only and the invention should in no way be construed as being limited to this Example but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

ATP induces a pronounced positive inotropic effect in a cardiac ventricular myocyte. However, the P2 receptor-effector mechanism that underlies this stimulatory cardiac action was not previously well understood. The objectives of the experiments described in this Example were to develop a cultured chick embryo ventricular myocyte as a model for cardiac P2 purinoceptor and to determine the

mechanism underlying the positive inotropic effect caused by contacting the purinoceptor with ATP. ATP caused an 89 ± 8.9 % increase in myocyte contractility. The efficacy and potency order of adenine nucleotides can be represented as follows: ATP>ADP>AMP>> adenosine. 2-Methylthio-ATP (2-MeSATP) was able to stimulate myocyte contractility, causing a maximal increase of contractility of 54 ± 2.6 %. α,β-methylene-ATP was not able to stimulate myocyte contractility.

Although UTP potently stimulates phosphoinositide hydrolysis, it had only a modest positive inotropic effect, causing a 27 ± 7 % maximal increase in myocyte contractility. The positive inotropic response stimulated by 2-MeSATP does not require the activity of phospholipase C (PLC). Instead, the effect of UTP on contractility appears to be mediated via a 2-MeSATP-sensitive P2 receptor. A PLC inhibitor, designated U-73122, had no effect on the positive inotropic response stimulated by 2-MeSATP, as indicated in Figure 6. This observation provides further evidence that PLC does not mediate the inotropic effect of 2-MeSATP. A cyclic AMP-independent calcium entry-stimulating mechanism appears to underlie a direct coupling of the receptor to stimulation of myocyte contractility. This new PLC- and cAMP-independent positive inotropic mechanism represents a target for developing novel positive inotropic therapeutics.

The materials and methods used in the experiments presented in the Experimental Example are now described.

Embryonic chick eggs were obtained from Spafas Inc. (Storrs, Conn). A cAMP radioimmunoassay kit was obtained from Amersham (Arlington Heights, IL). ^{3}H-leucine, ^{3}H-myoinositol, an $InsP_3$ radioreceptor assay kit, and ^{45}Ca were obtained from Dupont (Boston, MA). Adenosine, ADP, AMP, α,β-methylene-ATP, β,γ-methylene-ATP, and UTP were obtained from Sigma Chemical Co. (St. Louis, MO). 2-MeSATP was obtained from Research Biochemical International (Natick, MA). U-73122, which is also called 1(6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5 -dione, and U-73343, which is also called

1(6-((17b-3-methoxyestra-1,3,5(10)-trien- 17-yl)amino)hexyl)-2,5-pyrrolidine-dione were obtained from BIOMOL (Plymouth Meeting, PA).

Preparation of Cultured Cardiac Cells

Ventricular cells were obtained from chick embryos and cultured 14 days *in ovo* as described (Barry et al., 1982, J. Physiol. 325:243-260; Liang et al., 1995, Circ. Res. 76:242-251). After neutralizing trypsin with a medium comprising horse serum, the ventricular cells were centrifuged and resuspended in a culture medium comprising 6% (v/v) fetal bovine serum, 40% (v/v) Medium 199 (GIBCO, Grand Island, NY), 0.1 % (w/v each) penicillin/streptomycin, and Hank's salts. The cultured ventricular cells were plated at a density of 400,000 cell per milliliter and cultivated in a humidified 1:19 (vol:vol) CO_2:air atmosphere at 37°C. Cultured cardiac myocytes grew to confluence on day 3 in culture and exhibited rhythmic spontaneous contraction.

Assessment of ^{45}Ca Uptake into Myocardial Cells and Cellular cAMP Level

Determination of ^{45}Ca uptake was performed as described (Liang et al., 1996, J. Biol. Chem. 271:18678-18685). Cultures were incubated with L-(3, 4, 5-^{3}H, N)-leucine (152.2 Curies per millimole) for 24 hours prior to ^{45}Ca uptake. Incorporation of ^{3}H-leucine into the cellular protein permitted normalization of ^{45}Ca content to milligrams of cell protein by determining ^{3}H content, using known methods. Following exposure to ^{45}Ca, cells were then washed free of ^{45}Ca by rinsing the cells four times with ice-cold buffer containing 5 millimolar HEPES buffer, 1 millimolar $CaCl_2$, 4 millimolar KCl, 0.5 millimolar $MgCl_2$ 0.5, 142 millimolar NaCl, and 1 millimolar lanthanum, at pH 7.35. This washing procedure removed more than 99% of an extracellular marker, ^{51}Cr-EDTA, and effected substantially complete removal of extracellular ^{45}Ca. Uptake of ^{45}Ca was quantitated for 90 seconds. Cells were solubilized for 2 hours in a solution comprising 1% (w/v) sodium dodecyl sulfate and 10 millimolar sodium borate. Aliquots of the solution containing solubilized cells were assayed for radioactivity and protein content. cAMP was extracted by addition of one-tenth of the aliquot volume of 1 normal HCl to the media and boiling the mixture for 10 minutes. Extracted cAMP was assayed according to a described

radioimmunoassay method (Liang et al., 1995, Circ. Res. 76:242-251, Amersham, Arlington Heights, ILL). The effect of P2 receptor agonist on cyclic AMP accumulation was determined to be linear for at least 10 minutes, after which time the concentration of cAMP was assessed in the sample.

Determination of Contractile Amplitude

Contractile amplitude of cultured cardiac myocytes was assessed using an optico-video motion detection system as described (Barry et al., 1982, J. Physiol. 325:243-260; Liang et al., 1996, J. Biol. Chem. 271:18678-18685). Myocytes were paced at 2 Hertz by field stimulation using a platinum electrode, so that the effects of contractile amplitude were minimally affected by contractile period and the like. Myocytes were exposed a perfusion medium comprising an adenine nucleotide or analog, 4 millimolar HEPES buffer, 137 millimolar NaCl, 3.6 millimolar KCl, 0.5 millimolar $MgCl_2$, 1.1 millimolar $CaCl_2$, and 5.5 millimolar glucose at pH 7.4. Assessment of contractile amplitude was performed using only one cell per coverslip. Both the basal contraction amplitude and the amplitude measured during exposure to the adenine nucleotide or analog were determined.

Measurement of Phosphoinositide Response

Inositol phosphates were determined according to the method of Berridge et al (1983, Biochem. J. 212:473-482), as modified by Barnett et al (1990, Biochem. J. 271:437-442). Myocytes were pre-incubated with 5 millicuries per milliliter ^{3}H-myo-inositol for 24 hours, washed with Dulbecco's modified Eagle's medium comprising 15 millimolar LiCl (DMEM-Li), and incubated in DMEM-Li for 10 minutes at 37°C. The myocytes were then exposed to the adenine nucleotide or adenine analog.

Following this exposure, the myocytes were subjected to extraction using 1 milliliter of a solvent comprising a 1:2:0.05 (vol:vol:vol) mixture of chloroform:methanol:HCl to remove inositol phosphates from the myocytes. The solvent comprising inositol phosphates was applied to an anion exchange column (AGx8 resin, formate form, 1 milliliter bed volume) and $InsP_1$, InsP2, and $InsP_3$ were

eluted sequentially using a solution comprising 100 millimolar formic acid and 200 millimolar ammonium formate, a solution comprising 100 millimolar formic acid and 600 millimolar ammonium formate, and a solution comprising 100 millimolar formic acid and 1 M ammonium formate, respectively. The anion exchange column was calibrated with each inositol phosphate standard to confirm complete separation of $InsP_1$, InsP2, and $InsP_3$. Recovery of each inositol phosphate was greater than 95 %.

Inositol-1,4,5-trisphosphate Radioreceptor Assay

The effect of ATP receptor agonists on the $InsP_3$ level was quantitated using an $InsP_3$ radioreceptor assay. Growth media in which ventricular cells were grown was replaced with a solution comprising HEPES buffer, 1 millimolar $CaCl_2$, 4 millimolar KCl, and 0.5 millimolar $MgCl_2$ at pH 7.35. The cells were then exposed to ATP. The reaction was terminated by addition of 0.2 volumes of ice-cold trichloroacetic acid (TCA), which was removed by extraction with a solution comprising TCTFE (1,1,2-trichloro-1,2,2-trifluoroethane)-trioctylamine. $InsP_3$ in the aqueous phase was determined by competition with ^3H-$InsP_3$ for binding to the $InsP_3$ receptor supplied as a part of a kit, as described (Dupont, Boston, MA; Liang et al., 1996, J. Biol. Chem. 271:18678-18685).

The results of the experiments described in this Example are now described.

Characterization of the Positive Inotropic Response to ATP and Adenine Nucleotides

Treatment with ATP stimulated a marked increase in the contractile amplitude of myocytes, as illustrated in Figure 8A. The concentration of ATP which induced an approximately half-maximal increase in contractile amplitude (EC_{50}) was 0.16 ± 0.1 micromolar, the maximal increase in contractile amplitude being $89 \pm 8.9\%$ at about 3 micromolar ATP, as indicated in Figure 1.

ADP also induced a significant increase in myocyte contractility, the value of EC_{50} for ADP being 0.40 ± 0.3 micromolar. However, ADP was less efficacious than ATP for increasing contractile amplitude, the maximal increase in contractile amplitude being only $47 \pm 10.5\%$ at 10 micromolar ADP.

AMP and adenosine were less effective in stimulating myocyte contractility, the corresponding maximal increases in contractile amplitude being 10 ± 4.3% and 16 ± 3.7%, respectively. These results indicate that the inotropic effect of ATP is mediated by the P2 purinoceptor, rather than the P_1 purinoceptor.

Adult rat ventricular myocytes were isolated, and the effects of suramin and PPADS on 2-MeSATP-stimulated positive inotropic responses of those myocytes were determined. As indicated in Figures 9A and 9B, the presence of 100 micromolar suramin or 100 micromolar PPADS did not significantly affect 2-MeSATP-stimulated inotropic responses of myocytes.

To characterize the subtype of P2 purinoceptor that mediates the positive inotropic response of myocytes to ATP, a number of P2 receptor subtype-selective agonists were tested. The P2 receptor agonist 2-methylthio-ATP (2-MeSATP) caused a large increase in the contractile amplitude, as illustrated in Figure 8B. The value of EC_{50} for 2-MeSATP was 0.06 ± 0.05 micromolar. α,β-methylene-ATP and β,γ-methylene-ATP, which are agonists of some of the P2X receptors, were ineffective at stimulating myocyte contractility, as illustrated by the data presented in Figure 2. UTP, which is capable of activating the UTP-sensitive P2Y receptor, had a modest stimulatory effect on myocyte contractility, the value of EC_{50} for UTP being 0.3 ± 0.1 micromolar and the maximal increase in contractile amplitude for UTP being 27 ± 7 %. These results are consistent with a role of an UTP-sensitive P2Y receptor in mediating the positive inotropic response of myocytes to treatment with ATP. Because 2-MeSATP is a potent agonist of some of the P2X receptors, such as the $P2X_2$, $P2X_4$, $P2X_5$, and $P2X_6$ subtypes, it is possible that a P2X receptor can also mediate the ATP-induced positive inotropic effect in cardiac myocytes.

Subtype of Cardiac P2 Purinoceptor Coupled to Stimulation of Phosphatidyl-Inositol Hydrolysis

Because P2Y receptors can be coupled to activation of PIP2-PLC with consequent stimulation of phosphatidyl inositol (PI) hydrolysis (Harden et al., 1995, Annu. Rev. Pharmacol. Toxicol. 35:541-579), it was determined whether a

UTP-sensitive cardiac P2Y receptor is coupled to stimulation of PIP2-PLC and whether a resulting increase in PIP2-PLC activity mediates the positive inotropic response observed in cardiac myocytes.

Treatment of cardiac myocytes with ATP caused a significant increase in the intracellular levels of $InsP_1$, InsP2, and $InsP_3$, as depicted in Figure 3A. After 30 minutes of exposure of myocytes to ATP, there was a nearly six fold (570 ± 110%) increase in the intracellular concentration of total inositol phosphates ($InsP_{1-3}$). Half of the maximal increase in inositol phosphate concentration was achieved by treating the myocytes with an ATP concentration of 15 ± 10 micromolar, as illustrated in Figure 3B. The increase in the inositol 1,4,5-trisphosphate isomer was confirmed by using an inositol 1,4,5-trisphosphate radioreceptor assay. The basal concentration of the inositol 1,4,5-trisphosphate isomer was 42 ± 6 picomoles/milligram; in the presence of ATP the concentration of the inositol 1,4,5-trisphosphate isomer was 96 ± 4 picomoles/ milligram. The increase in $InsP_3$ concentration was transient, peaking 45 seconds after ATP treatment. Treatment of cardiac myocytes with UTP was also coupled to a pronounced stimulation of inositol phosphate production. Myocytes treated with 300 micromolar UTP experienced an increase in total inositol phosphates of 500 ± 90 %, and half-maximal inositol phosphate concentration increase was experienced by cells treated with 11 ± 10 micromolar UTP, as illustrated by the data depicted in Figure 3B. Neither the ATP- nor the UTP-stimulated PI response was attenuated by prior treatment of the myocytes with 5 nanograms pertussis toxin per milliliter over 24 hours, a treatment protocol that caused complete ADP-ribosylation of Gi by endogenous NAD^+ in these cultures (Liang et al., 1995, Circ. Res. 76:242-251; Liang et al., 1996, J. Biol. Chem. 271:18678-18685).

Since ATP is a potent agonist of both P2Y and P2X receptors, it is possible that the positive inotropic effect of ATP is mediated by both a PIP2-PLC-coupled, UTP-sensitive P2Y receptor and a phospholipase C-independent P2X or P_{2Y} receptor. To test this hypothesis, the effect of UTP and 2-MeSATP on PI hydrolysis was examined. UTP was as effective as ATP in stimulating PI hydrolysis,

while 2-MeSATP caused only a small increase in PI hydrolysis (52 ± 9 %). The P2X receptor-selective agonists α,β-methylene-ATP and β,γ-methylene-ATP were ineffective in stimulating PI hydrolysis. These data are consistent with the idea that a UTP-sensitive cardiac P2Y receptor is closely coupled to the activation of PIP2-PLC, and that a separate 2-MeSATP-sensitive P2 receptor is potently coupled to stimulation of myocyte contractility but is inefficiently coupled to PIP2-PLC activity. Alternatively, a P2X or P2Y receptor, activated by 2-MeSATP, may be selectively coupled to stimulation of myocyte contractility, while the UTP-sensitive P2Y receptor is coupled only to PIP2-PLC activity, which activity has no effect on the myocyte contractility. If the latter hypothesis is correct, then the stimulatory effect of 2-MeSATP on the PLC activity is due to its agonist activity at the PLC-coupled P2Y receptor, and the positive inotropic effect of UTP is due to its agonist activity at a 2-MeSATP-sensitive P2 purinoceptor. To provide further evidence for this conclusion, a number of cross-desensitization experiments were carried out.

Role of PIP2-PLC in Mediating the P2 Receptor Agonist-induced Positive Inotropic Response

UTP- and 2-MeSATP-induced PI hydrolysis was reduced by incubating myocytes for 80 minutes in a medium comprising 100 micromolar UTP, removing the UTP, and then assessing UTP- or 2-MeSATP- induced PI hydrolysis, as depicted in Figure 4. However, incubation of myocytes with 100 micromolar 2-MeSATP for 80 minutes prior to UTP- or 2-MeSATP induction of PI hydrolysis had no effect on the basal level of inositol phosphates, $InsP_{1-3}$ levels being 9883 ± 320 units in cells which were not incubated with 2-MeSATP and 9214 ± 410 units in cells incubated with 2-MeSATP. Incubation of myocytes with 100 micromolar 2-MeSATP also had no effect on PI hydrolysis induced by addition of either UTP ($InsP_{1-3}$ levels being 45313 ± 1820 units in myocytes not incubated with 2-MeSATP and 46576 ± 1694 units in myocytes incubated with 2-MeSATP) or 2-MeSATP ($InsP_{1-3}$ levels being 15842 ± 2010 units in myocytes not incubated with 2-MeSATP and 14265 ± 1902 units in myocytes incubated with 2-MeSATP).

Next, the role of PLC-coupled P2Y receptor in mediating the positive inotropic response was examined. An 80 minute exposure of myocytes to 100 micromolar 2-MeSATP caused a significant reduction of the ATP-induced positive inotropic response, which was manifested as a 73 ± 4 % decrease in the extent of stimulation of myocyte contractility. 2-MeSATP-exposed myocytes also exhibited a diminished positive inotropic response to 2-MeSATP, and exhibited virtually no increase in myocyte contractile amplitude in response to UTP, as depicted in Figures 5A and 5B. However, a 90 minute exposure to 100 micromolar UTP had no effect on 2-MeSATP- or UTP-induced increase in myocyte contractility, as depicted in Figures 5C and 5D. These data are consistent with the hypothesis that a P2Y receptor, activated to a much greater extent by UTP than by 2-MeSATP, is coupled to stimulation of PIP2-PLC, and that a P2X or P2Y receptor activated to a much greater extent by 2-MeSATP than by UTP is coupled to stimulation of myocyte contractility.

The aminosteroid U-73122, which is a known inhibitor of the receptor-mediated activation of PLC, was used to further determine whether PLC plays a role in 2-MeSATP-stimulated increase in myocyte contractility. Neither 1 micromolar nor 10 micromolar U-73122 had an effect on the 2-MeSATP-stimulated increase in myocyte contractility, as depicted in Figure 6. 10 micromolar U-73122, a concentration known to inhibit completely the PLC-mediated PI hydrolysis (Smith et al., 1990, J. Pharmacol. Exp. Ther. 253:688-697; Thompson et al., 1991, J. Biol. Chem. 266:23856-23862), caused a slight depression of the myocyte contractility, but did not affect 2-MeSATP-induced stimulation of contractility, the increase in contractile amplitude of myocytes treated with 2-MeSATP and U-73122 being 48.4 ± 8 %, a value comparable to that observed using 2-MeSATP alone. The inactive structural analog of U-73122, designated U-73343, also had no effect on 2-MeSATP-stimulated increase in myocyte contractility.

cAMP-independent ^{45}Ca Entry Underlies the P2 Receptor-Mediated Positive Inotropic Response

ATP can induce an increase in myocyte contractile amplitude (Berridge et al., 1983, Biochem. J. 212:473-482) as well as an increase in calcium entry and an increase in the level of cytosolic calcium (Christie et al., 1992, J. Physiol. 445:369-388; DeYoung et al., 1989, Am. J. Physiol. 257(Cell Physiol. 26):C750-C758; Scamps et al., 1992, J. Gen. Physiol. 100:675-701). Both 2-MeSATP and ATP caused a pronounced increase in the transsarcolemmal uptake of ^{45}Ca. However, neither α,β-methylene-ATP, as indicated in Figure 7, nor β,γ-methylene-ATP had any significant stimulatory effect on ^{45}Ca uptake by myocytes. Neither 2-MeSATP nor ATP was able to stimulate cAMP accumulation, the basal cAMP level being 12.2 ± 2 picomoles/milligram and cAMP levels in the presence of 2-MeSATP and ATP being 13.1 ± 1.6 and 13.2 ± 1.1, respectively. No other P2 receptor agonist, including UTP, α,β-methylene-ATP, or β,γ-methylene-ATP was able to induce cAMP accumulation in myocytes. As a positive control, cells were treated with isoproterenol, and a 6.7 ± 1.4- fold increase in the level of cAMP was observed. These data indicate that a cAMP-independent calcium entry-stimulating mechanism mediates the P2 receptor agonist-induced stimulation of myocyte contractility.

Cultured chick embryo ventricular myocytes are as a useful experimental model for a number of receptor-effector systems, including the P_1 (adenosine) receptor. Because these myocytes remain stable in culture for at least three days, various interventions can be performed, such as desensitization studies and equilibration of ^{3}H-myo-inositol within the myocyte pool to enable examination of PI hydrolysis. The feasibility of preparing relatively large number of myocytes also facilitates the biochemical determination of phosphoinositide levels. The stability of the cultured myocytes enables reliable and reproducible determination of changes in contractility in response to various agonists.

Thus, the cultured ventricular myocytes described herein represent a model system in which a stable and reproducible ATP-induced positive inotropic

response facilitates full characterization of the receptor(s) involved as well as studies of the mechanism underlying the receptor(s) physiological effects and of compounds capable of interfering with that mechanism.

Adenine nucleotides caused a pronounced stimulation of the myocyte contractility with the order of efficacy being ATP > ADP > AMP >> adenosine. This observation is consistent with the hypothesis that the positive inotropic response of myocytes to adenine nucleotide treatment is mediated by a P2 purinoceptor. That the order of potency and efficacy is ATP > 2-MeSATP > UTP > (α,β-methylene-ATP or β,γ-methylene-ATP) is consistent with the hypothesis that the subtype of P2 receptor mediating the positive inotropic effect is a P2Y receptor or a 2-MeSATP-sensitive P2X receptor, such as $P2X_2$, $P2X_4$, $P2X_5$, or $P2X_6$ receptor (Burnstock et al., 1996, Drug Develop. Res., 38:67-71).

Because UTP exhibits a significant positive inotropic effect, it was possible that a UTP-sensitive P2 receptor, either one of the known P2Y receptors or a novel receptor, also mediated some of the ATP-stimulated positive inotropic effect (Burnstock et al., 1996, Drug Develop. Res., 38:67-71; Harden et al., 1995, Annu. Rev. Pharmacol. Toxicol. 35:541-579). Although the concentrations of ATP and UTP required for half maximal stimulation of PI hydrolysis were higher than those for the increase in contractile amplitude, 1 micromolar ATP or UTP was able to induce a significant increase in intracellular inositol phosphate levels (85 ± 10 % increase using ATP and 90 ± 15 % increase using UTP). Because UTP causes a pronounced increase in the level of $InsP_{1-3}$ and because $InsP_3$ is known to stimulate release of calcium from the sarcoplasmic reticulum (Vites et al., 1990, Am. J. Physiol. 258(Heart Circ. Physiol. 27):H1745-H1752; Vites et al., 1992, Am. J. Physiol. 262(Heart Circ. Physiol. 31):H268-H277), it was possible that the receptor-mediated increase in $InsP_3$ caused the positive inotropic effect observed in UTP-treated cells. Because 2-MeSATP caused only a modest stimulation of inositol phosphate production, the 2-MeSATP-sensitive P2 receptor may be coupled directly to stimulation of myocyte contractility independent of any increase in $InsP_{1-3}$ production. According to this hypothesis, ATP

induced its positive inotropic effect by activating both a PLC-coupled P2Y receptor and a 2-MeSATP-sensitive P2 receptor. Alternatively, the positive inotropic effect induced by UTP is due to its agonist activity, although modest, at the 2-MeSATP-sensitive P2 purinoceptor, and the stimulatory effect induced by 2-MeSATP on $InsP_{1-3}$ level is caused by the cross-activity or 2-MeSATP at the UTP-sensitive P2Y receptor.

Two lines of evidence support this latter hypothesis. First, treatment of myocytes with UTP prior to treatment of the myocytes with UTP or 2-MeSATP decreased stimulation of $InsP_{1-3}$ production. On the other hand, treatment of myocytes with 2-MeSATP prior to treatment of the myocytes with UTP or 2-MeSATP had no effect on stimulation of $InsP_{1-3}$ production. These observations suggest that only the UTP-sensitive P2Y receptor is coupled to inositol phosphate production. Second, incubation of myocytes with 2-MeSATP prior to treatment of the myocytes with UTP or 2-MeSATP significantly reduced the positive inotropic response otherwise induced by this treatment. Incubation of myocytes with UTP prior to treatment of the myocytes with UTP or 2-MeSATP had no effect on UTP- or 2-MeSATP-stimulated myocyte contractility. These observations suggest that only the 2-MeSATP-sensitive P2 purinoceptor is coupled to the stimulation of myocyte contractility.

The observation that ATP can stimulate PI hydrolysis in cultured chick ventricular myocytes is similar to the observations made using mouse (Yamada et al., 1992, Circ. Res. 70:477-485) and rat (Leggssyer et al., 1988, J. Physiol. 401:185-199) ventricular myocytes. The experiments presented in this Example demonstrate that the stimulatory effect of ATP is mediated via an UTP-sensitive P2Y receptor. In contrast to previous suggestions (Leggssyer et al., 1988, J. Physiol. 401:185-199), the present observations suggest that the positive inotropic response of myocytes to ATP, such as that mediated via the 2-MeSATP-sensitive P2 receptor, occurs independently of PLC activation. Formation of $InsP_3$ is not necessary to induce the 2-MeSATP-stimulated positive inotropic response. The conclusions described herein were further supported

by the observation that 1 micromolar 2-MeSATP caused a maximal positive inotropic effect, but that this concentration of 2-MeSATP had no effect on intracellular $InsP_{1-3}$ levels. Further evidence that PLC is not involved in the 2-MeSATP-inducible inotropic response was provided by observing that U-73122, a known PLC inhibitor, had no effect on 2-MeSATP-induced myocyte contractility even at an inhibitor concentration of 10 micromolar, as indicated in Figure 9C.

ATP agonists were able to cause a significant stimulation of transsarcolemmal calcium entry in myocytes. However, none of the ATP agonists used in the experiments presented herein caused an increase in cellular cAMP content. This observation is similar to that made using rat ventricular myocytes (Scamps et al., 1992, J. Gen. Physiol. 100:675-701). The order of efficacy of adenine nucleotides for stimulating calcium entry, namely ATP > 2-MeSATP > UTP > α,β-methylene-ATP, is similar to the order of efficacy of the same compounds in stimulating myocyte contractility. Thus, these data suggest that a cAMP-independent, calcium entry-stimulating mechanism underlies the 2-MeSATP-sensitive P2 purinoceptor-mediated increase in myocyte contractile amplitude.

The present observations are compatible with findings that the classical P2Y agonist, 2-MeSATP, stimulates calcium entry via both a non-selective cation channel and a L-type calcium channel (Scamps et al., 1990, Circ. Res 67:1007-1016; Scamps et al., 1994, Br. J. Pharmacol. 113:982-986) and an increase in calcium transients in rat ventricular myocytes (Bjornsson et al., 1989, Eur. J. Biochem. 186:395-404). It is unlikely that ATP-induced acidification, with consequent stimulation of cytosolic calcium level, contributes to the positive inotropic effect of ATP, because ATP-induced acidification requires the presence of a Mg-ATP complex at a concentration 100 fold higher than that necessary to cause increased myocyte contractility.

Recombinant myocytes

Recombinant myocytes were made by transfecting embryonic chick myocytes using either a pcDNA3 vector alone or a pcDNA3 vector comprising a

recombinant gene encoding the $P2X_4$ purinoceptor. The pcDNA3 vector comprising the recombinant gene was made by inserting a nucleic acid encoding the $P2X_4$ purinoceptor (Garcia-Guzman et al., 1996, FEBS Lett. 388:123-127) into the multiple cloning site of a commercially-available pcDNA3 vector (Invitrogen, Carlsbad, CA). The embryonic chick ventricular myocytes were transfected using the vector using the modified calcium phosphate transfection method described by Xu et al. (1992, Nucl. Acids Res. 20:6425-6426). As indicated in Figure 10, the presence of the recombinant gene in myocytes approximately doubled the magnitude of the 2-MeSATP-induced calcium influx. As indicated in Figure 11, the presence of the recombinant gene in myocytes also significantly increased the magnitude of contractile magnitude induced by treatment of the myocytes with 2-MeSATP. These observations indicate that the $P2X_4$ purinoceptor is a purinoceptor which mediates induction of contractility of myocytes by adenine nucleotides such as ATP and 2-MeSATP.

Identity of Relevant P2 Purinoceptors

The identity of P2 purinoceptors that mediate the increase in myocyte contractility is now described. The facts that some of the recently cloned P2X receptors are potently activated by 2-MeSATP and that mRNAs encoding these receptors are expressed in the heart (Garcia-Guzman et al., 1996, FEBS Lett. 388:123-127; Soto et al., 1996, Proc. Natl. Acad. Sci. USA 93:3684-3688) support the conclusion that a P2X receptor mediates the positive inotropic effect of ATP. The P2X receptor is unlikely to be either the $P2X_1$ receptor or the $P2X_3$ receptor because the current mediated by the $P2X_1$ and $P2X_3$ receptors desensitizes completely within a few seconds (Collo et al., 1996, J. Neurosci. 16:2495-2507) and because these two P2X receptors can be activated by α,β-methylene-ATP.

Overall, the present data demonstrate that a novel cAMP- and PLC-independent calcium entry pathway, likely mediating the direct coupling of a P2 purinoceptor to stimulation of myocyte contractility, exists in the intact cardiac cell. The cAMP- and PLC-independent receptor is not likely to be a P2Y receptor, because the underlying positive inotropic mechanism does not involve the action of PLC or

cAMP, whereas all of the known P2Y receptors either stimulate PLC activity or inhibit adenylyl cyclase activity.

Thus, the stimulatory receptor is one or both of the $P2X_4$ and $P2X_6$ receptors. This hypothesis is supported by the observation that of the known P2X receptors, only the $P2X_4$, $P2X_5$, and $P2X_6$ receptors can be activated by 2-MeSATP. Of these three receptors, the function of only the $P2X_5$ receptor is sensitive to inhibition by suramin, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), and reactive blue-2. The functions of the $P2X_4$ and $P2X_6$ receptors are not sensitive to inhibition by these compounds. It has been discovered that the function of the P2 purinoceptor responsible for stimulating myocyte contractility is not sensitive to inhibition by suramin, PPADS, or reactive blue-2, but can be activated by 2-MeSATP. Furthermore, mRNA molecules encoding each of the $P2X_4$ and $P2X_6$ receptors are known to be expressed in the heart. Therefore, the $P2X_4$ and $P2X_6$ represent novel targets for the development of new positive inotropic therapeutics.

Cardiac ventricular myocytes were isolated from adult rats. The contractile amplitude of these myocytes was determined in the presence of both 200 nanomolar trinitrophenyl-ATP (TNP-ATP) and 3 micromolar 2-MeSATP and, separately, in the presence of 3 micromolar 2-MeSATP. TNP-ATP is an antagonist of both $P2X_1$ and $P2X_3$ purinoceptors. The contractile amplitude of the myocytes was approximately the same in the presence and absence of TNP-ATP. The fact that TNP-ATP failed to attenuate the positive inotropic response of the myocytes to 2-MeSATP indicates that the inotropic response of the myocytes to the presence of 2-MeSATP was not attributable to the $P2X_1$ purinoceptor or to the $P2X_3$ purinoceptor.

The ability of suramin, a non-selective antagonist of most P2X and P2Y purinoceptors other than $P2X_4$ to enhance the increase in contractility of adult rat and chick embryo myocytes in the presence of 2-MeSATP. This results indicates that cardiac myocyte contractility can be enhanced to a greater degree in the presence of both a $P2X_4$ (or $P2X_6$) purinoceptor agonist and an antagonist of a purinoceptor other than $P2X_4$ or $P2X_6$ than in the presence of a $P2X_4$ (or $P2X_6$) purinoceptor agonist

alone. Preferably, a $P2X_4$ purinoceptor agonist and an antagonist of a purinoceptor other than $P2X_4$ are used in combination.

Cultured chick embryo ventricular myocytes were transfected using a pcDNA3 vector comprising a nucleic acid encoding a $P2X_5$ purinoceptor. Treatment of these myocytes with 2-MeSATP (3 micromolar) did not result in greater stimulation of contractility (15 ± 4.7%) as compared with the ability of the same concentration of 2-MeSATP to stimulate contractility of cultured ventricular myocytes that had been transfected with a pcDNA3 vector which did not encode a $P2X_5$ purinoceptor (16.5 ± 1%). Cultured chick embryo ventricular myocytes transfected using a pcDNA3 vector comprising a nucleic acid encoding a $P2X_4$ purinoceptor exhibited a 45.5 ± 6% stimulation of contractility in response to 3 micromolar 2-MeSATP.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

What is claimed is:

1. A recombinant myocyte comprising at least one introduced nucleic acid encoding at least one P2 purinoceptor.

2. The recombinant myocyte of claim 1, wherein said P2 purinoceptor is selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, a $P2X_4$ purinoceptor, a $P2X_5$ purinoceptor, and a $P2X_6$ purinoceptor.

3. The recombinant myocyte of claim 1, wherein said P2 purinoceptor is selected from the group consisting of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor.

4. The recombinant myocyte of claim 1, wherein said myocyte is selected from the group consisting of a chicken embryonic ventricular myocyte and a rat ventricular myocyte.

5. A method of determining whether a compound enhances cardiac contractility, said method comprising assessing the contractile amplitude of a recombinant myocyte in the presence and absence of the compound, wherein said myocyte comprises at least one first introduced nucleic acid encoding at least one of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor, whereby an increase in the contractile amplitude of said myocyte in the presence of the compound, relative to the contractile amplitude of said myocyte in the absence of the compound, is an indication that the compound enhances cardiac contractility.

6. The method of claim 5, wherein said myocyte is selected from the group consisting of a chicken embryonic ventricular myocyte and a rat ventricular myocyte.

7. The method of claim 5, further comprising assessing the contractile amplitude of a non-recombinant myocyte in the presence and absence of the compound, whereby if the difference between the contractile amplitude of said recombinant myocyte in the presence of the compound and the contractile amplitude of said recombinant myocyte in the absence of the compound is greater than the difference between the contractile amplitude of said non-recombinant myocyte in the

presence of the compound and the contractile amplitude of said non-recombinant myocyte in the absence of the compound, then the effect of the composition on contractile amplitude is attributable to said at least one purinoceptor.

8. A method of determining whether a compound enhances cardiac contractility, said method comprising assessing calcium uptake by a recombinant myocyte in the presence and absence of the compound, wherein said myocyte comprises at least one first introduced nucleic acid encoding at least one of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor, whereby an increase in calcium uptake by said myocyte in the presence of the compound, relative to calcium uptake by said myocyte in the absence of the compound, is an indication that the compound enhances cardiac contractility.

9. The method of claim 8, further comprising assessing calcium uptake by a non-recombinant myocyte in the presence and absence of the compound, whereby if the difference between calcium uptake by said recombinant myocyte in the presence of the compound and calcium uptake by said recombinant myocyte in the absence of the compound is greater than the difference between calcium uptake by said non-recombinant myocyte in the presence of the compound and calcium uptake by said non-recombinant myocyte in the absence of the compound, then the effect of the composition on calcium uptake is attributable to said at least one purinoceptor.

10. A method of determining whether a compound enhances cardiac contractility, said method comprising

assessing the contractile amplitude of a first myocyte in the presence and absence of the compound, wherein said first myocyte comprises at least one first P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, and a $P2X_4/P2X_6$ heterodimer; and

assessing the contractile amplitude of a second myocyte in the presence and absence of the compound, wherein said second myocyte comprises said at least one first P2 purinoceptor and at least one introduced nucleic acid encoding at least one second P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor;

whereby if the difference between the contractile amplitude of said second myocyte in the presence of the compound and the contractile amplitude of said second myocyte in the absence of the compound is greater than the difference between the contractile amplitude of said first myocyte in the presence of the compound and the contractile amplitude of said first myocyte in the absence of the compound, then the compound enhances cardiac contractility.

11. The method of claim 10, wherein said second P2 purinoceptor is selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, and a $P2X_5$ purinoceptor.

12. A method of determining whether a compound enhances cardiac contractility, said method comprising

assessing calcium uptake by a first myocyte in the presence and absence of the compound, wherein said first myocyte comprises at least one first P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, and a $P2X_4/P2X_6$ heterodimer; and

assessing calcium uptake by a second myocyte in the presence and absence of the compound, wherein said second myocyte comprises said at least one first P2 purinoceptor and at least one introduced nucleic acid encoding at least one second P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor;

whereby if the difference between calcium uptake by said second myocyte in the presence of the compound and calcium uptake by said second myocyte in the absence of the compound is greater than the difference between calcium uptake by said first myocyte in the presence of the compound and calcium uptake by said first myocyte in the absence of the compound, then the compound enhances cardiac contractility.

13. The method of claim 12, wherein said second P2 purinoceptor is selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, and a $P2X_5$ purinoceptor.

14. A method of enhancing cardiac contractility in an animal, said method comprising administering to the animal a composition comprising at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, and an antagonist of a P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor, whereby cardiac contractility is augmented in the animal.

15. The method of claim 14, wherein at least one agent is selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist.

16. The method of claim 15, wherein at least one agent is 2-methylthio-ATP.

17. The method of claim 16, wherein said 2-methylthio-ATP is administered in an amount sufficient to effect a concentration of at least about 0.3 micromolar 2-methylthio-ATP in a cardiac tissue of the animal.

18. The method of claim 17, wherein said 2-methylthio-ATP is administered in an amount sufficient to effect a concentration of at least about 3 micromolar 2-methylthio-ATP in the cardiac tissue.

19. The method of claim 15, further comprising administering to the animal at least one antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

20. The method of claim 19, wherein said P2 purinoceptor is selected from the group consisting of a P2Y purinoceptor, a $P2X_1$ purinoceptor, a $P2X_2$ purinoceptor, a $P2X_3$ purinoceptor, and a $P2X_5$ purinoceptor.

21. The method of claim 20, wherein at least one antagonist is selected from the group consisting of suramin, reactive blue-2, and pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid.

22. The method of claim 21, wherein said at least one antagonist is administered in an amount sufficient to effect a concentration of at least about 30 micromolar antagonist in a cardiac tissue of the animal.

23. The method of claim 22, wherein said at least one antagonist is administered in an amount sufficient to effect a concentration of at least about 300 micromolar antagonist in the cardiac tissue.

24. The method of claim 14, wherein the animal is a mammal.

25. The method of claim 24, wherein the mammal is a human.

26. A pharmaceutical composition for enhancing cardiac contractility, said pharmaceutical composition comprising at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, and an antagonist of a P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

27. The pharmaceutical composition of claim 26, wherein at least one agent is selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist.

28. The pharmaceutical composition of claim 27, further comprising at least one antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

29. A kit comprising a first container containing at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist and a second container containing at least one antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

30. A method of treating an animal experiencing heart failure, said method comprising administering to the animal a composition comprising at least one agent selected from the group consisting of a $P2X_4$ purinoceptor agonist, a $P2X_6$ purinoceptor agonist, and an antagonist of a P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

31. The method of claim 30, wherein at least one agent is selected from the group consisting of a $P2X_4$ purinoceptor agonist and a $P2X_6$ purinoceptor agonist.

32. The method of claim 31, further comprising administering to the animal an antagonist of at least one P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

33. A kit for determining whether a compound enhances cardiac contractility, said kit comprising a recombinant myocyte comprising at least one introduced nucleic acid encoding at least one P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor and an instructional material which describes assessment of at least one of the contractile amplitude of said myocyte and calcium uptake by said myocyte.

34. A kit for determining whether a compound enhances cardiac contractility, said kit comprising

a first myocyte comprising at least one first P2 purinoceptor selected from the group consisting of a $P2X_4$ purinoceptor, a $P2X_6$ purinoceptor, and a $P2X_4/P2X_6$ heterodimer; and

a second myocyte comprising at least one introduced nucleic acid encoding at least one second P2 purinoceptor other than a $P2X_4$ purinoceptor or a $P2X_6$ purinoceptor.

35. A method of determining whether a compound decreases cardiac contractility, said method comprising assessing the contractile amplitude of a recombinant myocyte in the presence and absence of the compound, wherein said myocyte comprises at least one first introduced nucleic acid encoding at least one of a $P2X_4$ purinoceptor and a $P2X_6$ purinoceptor, whereby a decrease in the contractile amplitude of said myocyte in the presence of the compound, relative to the contractile amplitude of said myocyte in the absence of the compound, is an indication that the compound decreases cardiac contractility.

36. The method of claim 35, further comprising assessing the contractile amplitude of a non-recombinant myocyte in the presence and absence of the compound, whereby if the decrease in the contractile amplitude of said recombinant myocyte in the presence of the compound, relative to the decrease in the contractile

amplitude of said recombinant myocyte in the absence of the compound, is greater than the decrease in the contractile amplitude of said non-recombinant myocyte in the presence of the compound, relative to the decrease in the contractile amplitude of said non-recombinant myocyte in the absence of the compound, then the effect of the composition on contractile amplitude is attributable to said at least one purinoceptor.

37. A method of decreasing cardiac contractility in an animal, said method comprising administering to the animal a composition comprising at least one agent selected from the group consisting of a $P2X_4$ purinoceptor antagonist and a $P2X_6$ purinoceptor antagonist, whereby cardiac contractility is decreased in the animal.

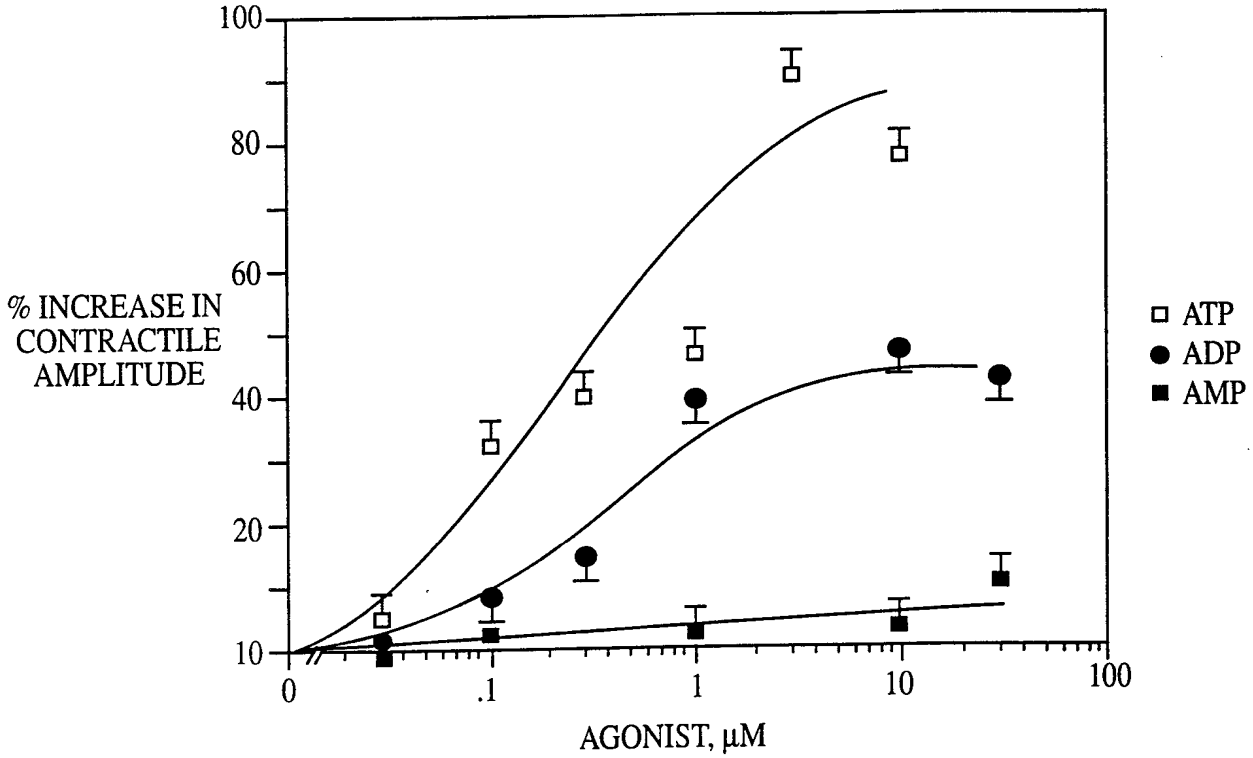
100
80
60
40
20
10
% INCREASE IN CONTRACTILE AMPLITUDE
0
.1
1
10
100
AGONIST, μM
ATP
ADP
AMP

Fig. 1A

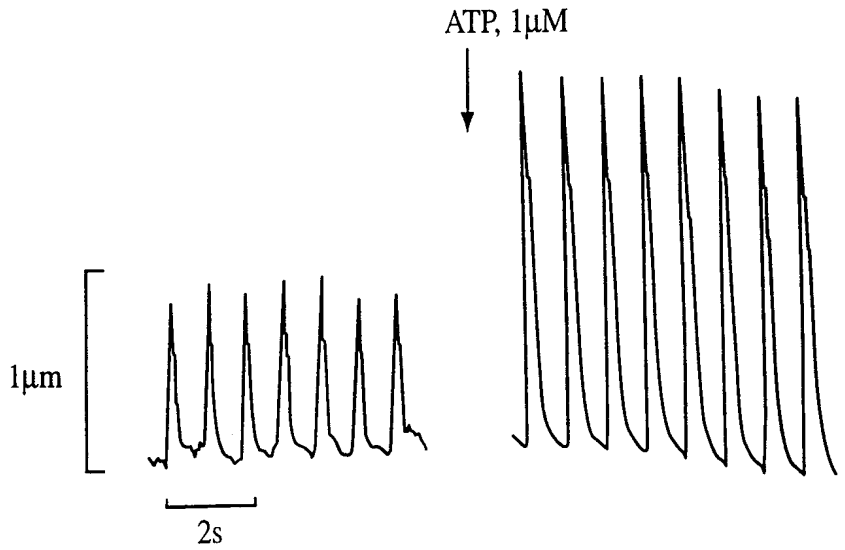
ATP, 1μM
1μm
2s

Fig. 1B

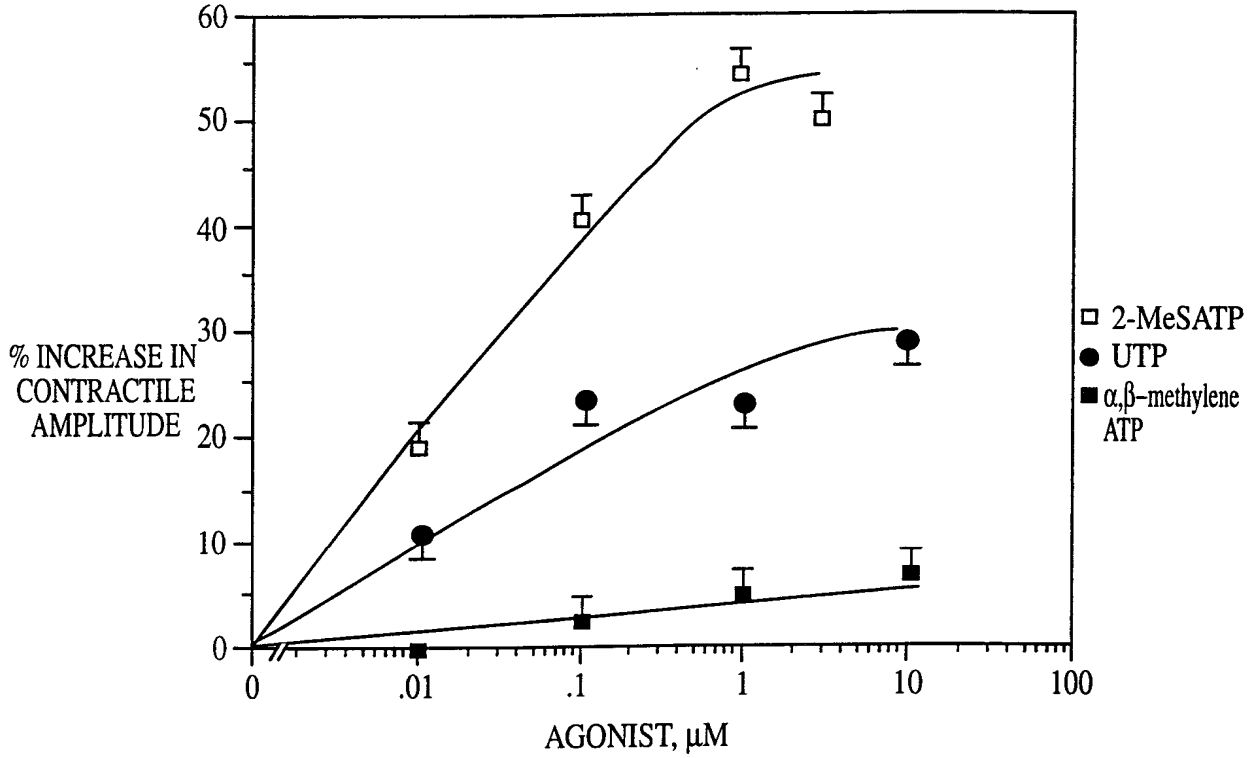
60
50
40
30
20
10
0
% INCREASE IN CONTRACTILE AMPLITUDE
0
.01
.1
1
10
100
AGONIST, μM
2-MeSATP
UTP
α,β-methylene ATP

Fig. 2

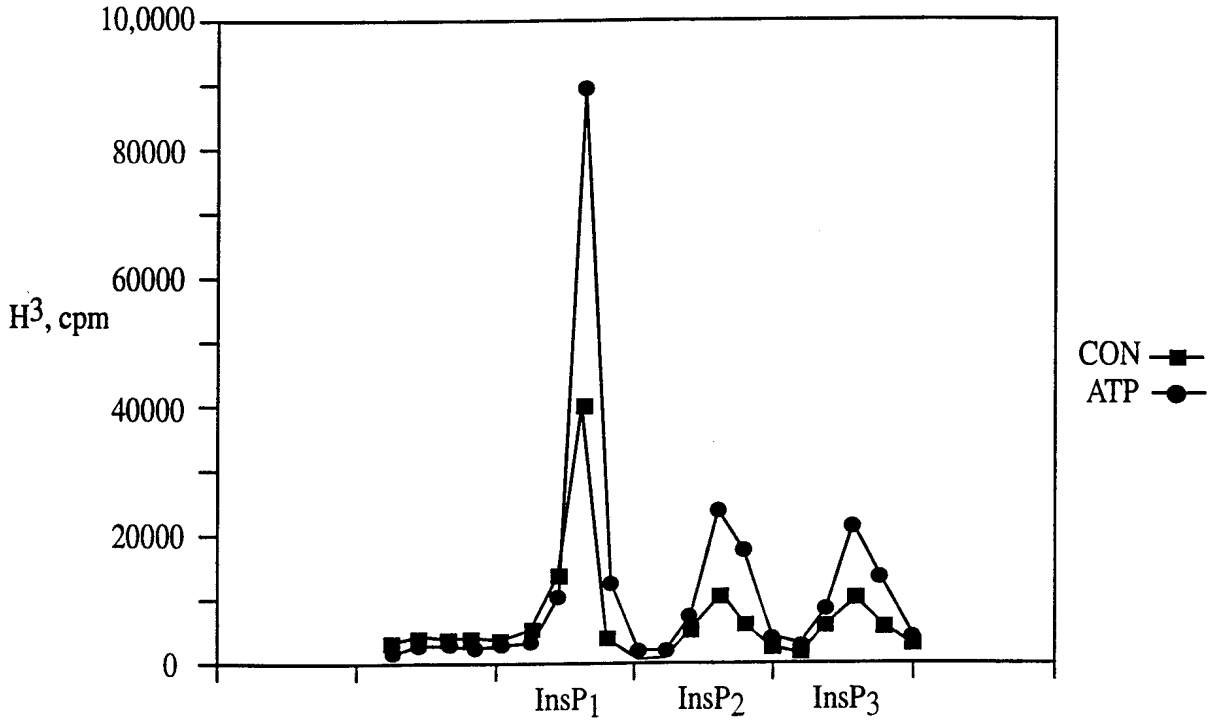
10,0000
80000
60000
40000
20000
0
H3, cpm
InsP1
InsP2
InsP3
CON
ATP

Fig. 3A

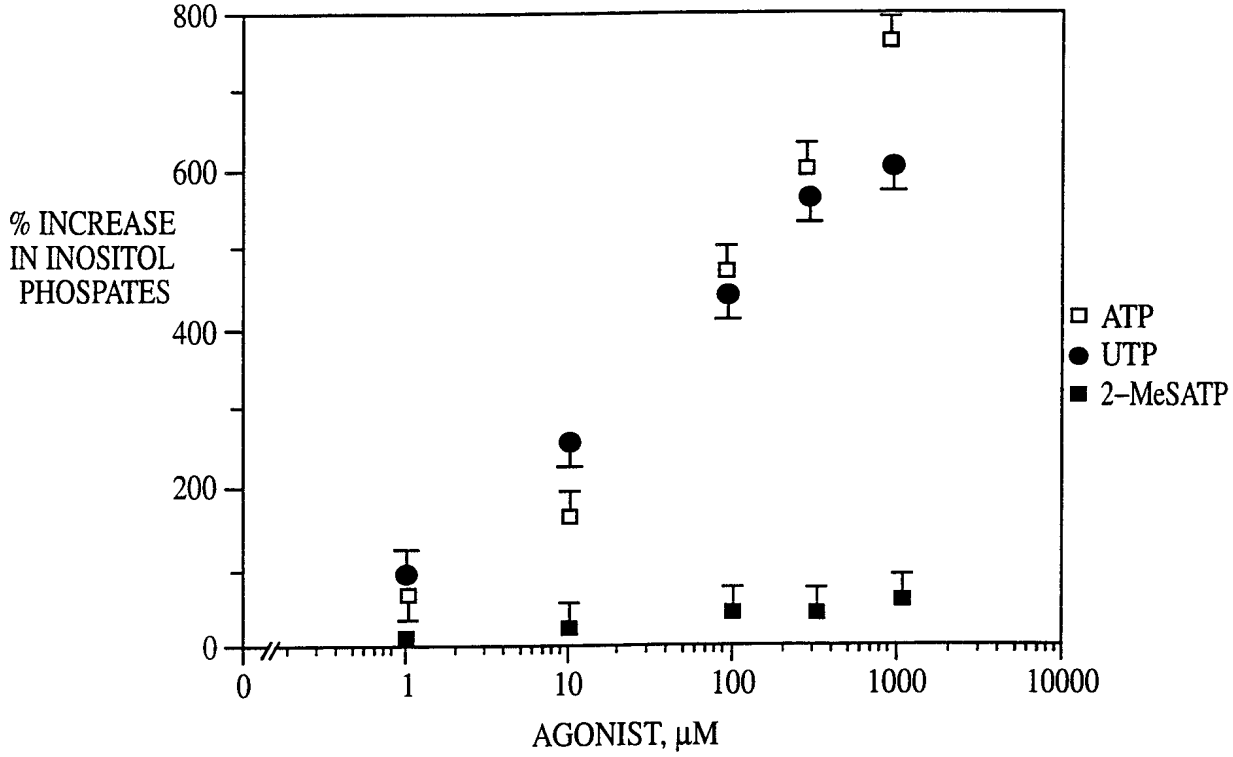
800
600
400
200
0
% INCREASE IN INOSITOL PHOSPATES
0
1
10
100
1000
10000
AGONIST, μM
ATP
UTP
2-MeSATP

Fig. 3B

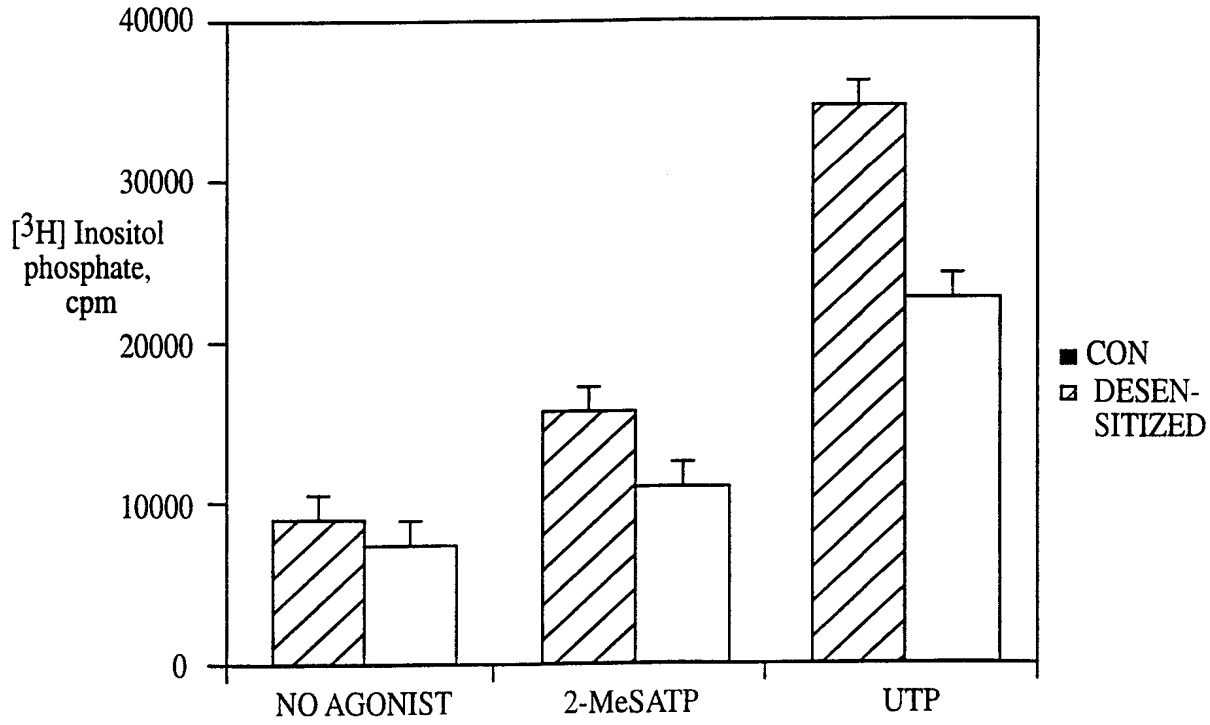
40000
30000
20000
10000
0
[3H] Inositol phosphate, cpm
NO AGONIST
2-MeSATP
UTP
CON
DESEN-SITIZED

FIG. 4

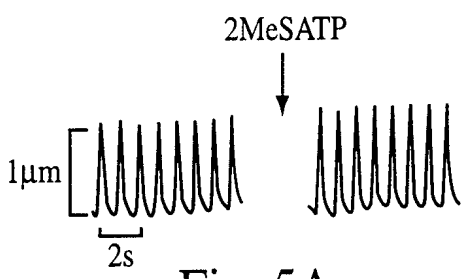

Fig. 5A

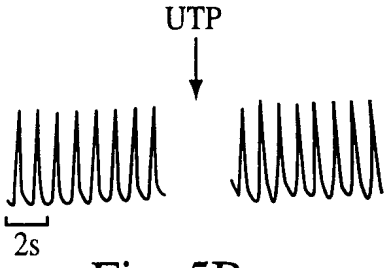

Fig. 5B

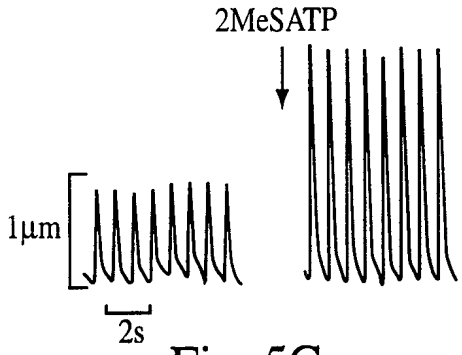

Fig. 5C

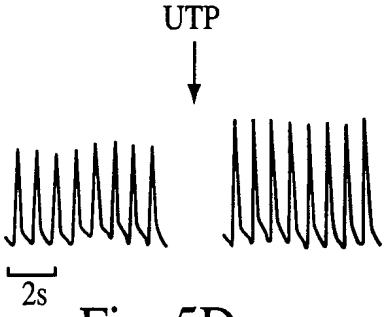

Fig. 5D

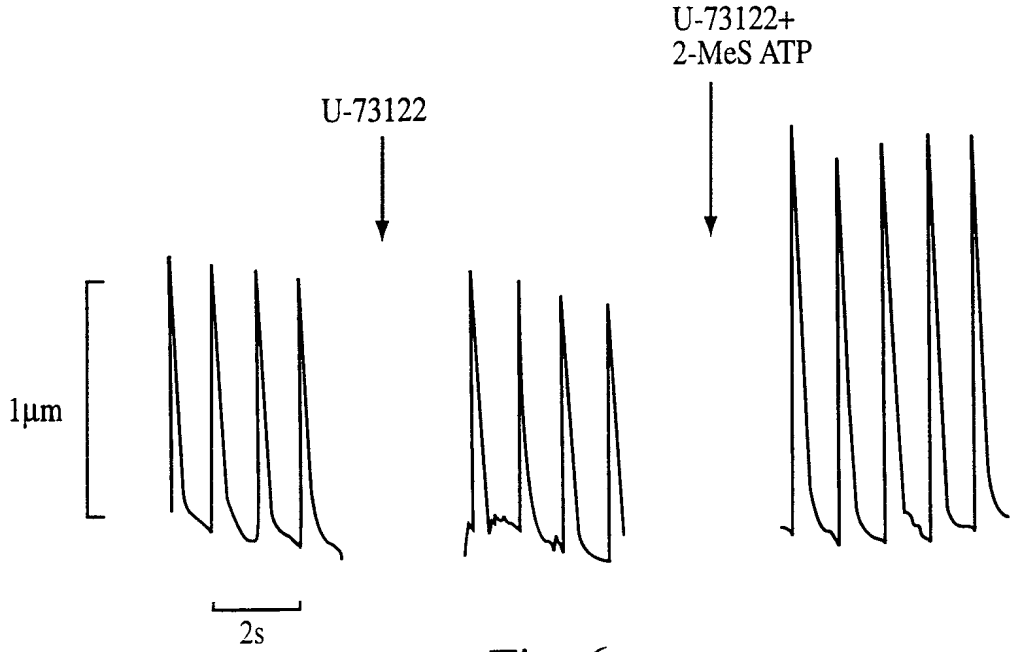

Fig. 6

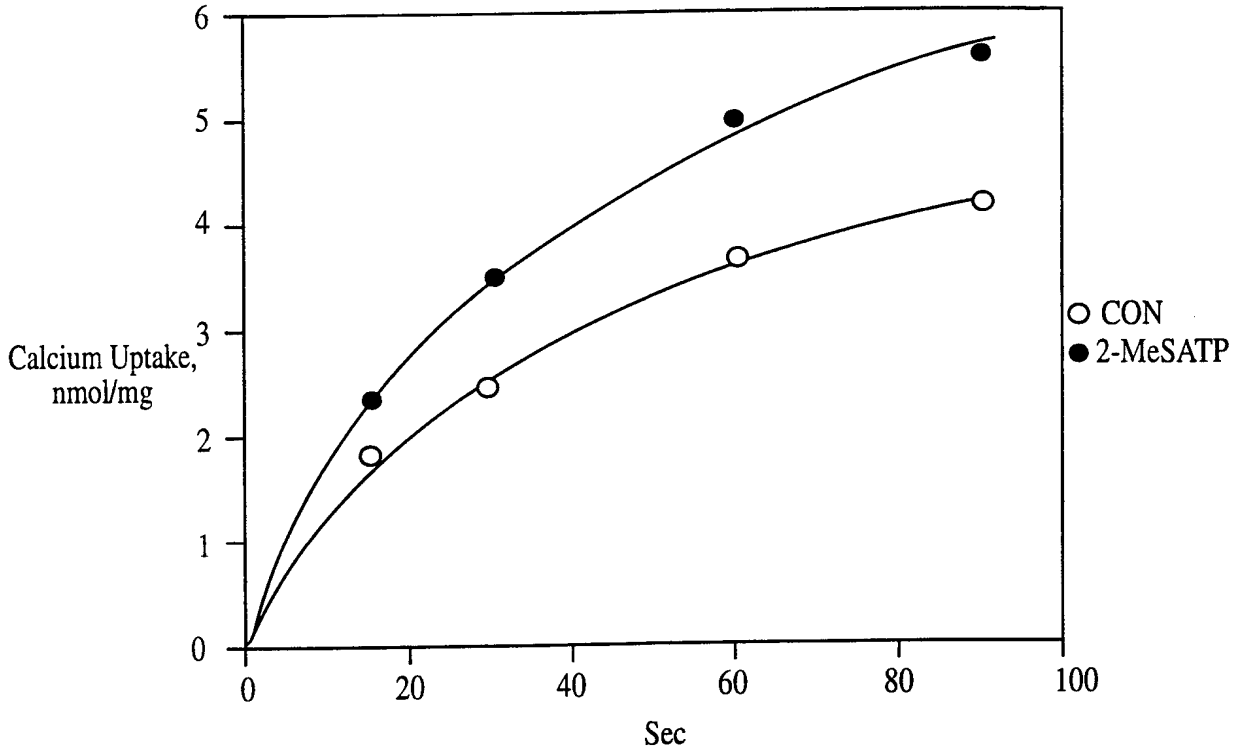
Calcium Uptake, nmol/mg
0
1
2
3
4
5
6
0
20
40
60
80
100
Sec
CON
2-MeSATP

Fig. 7A

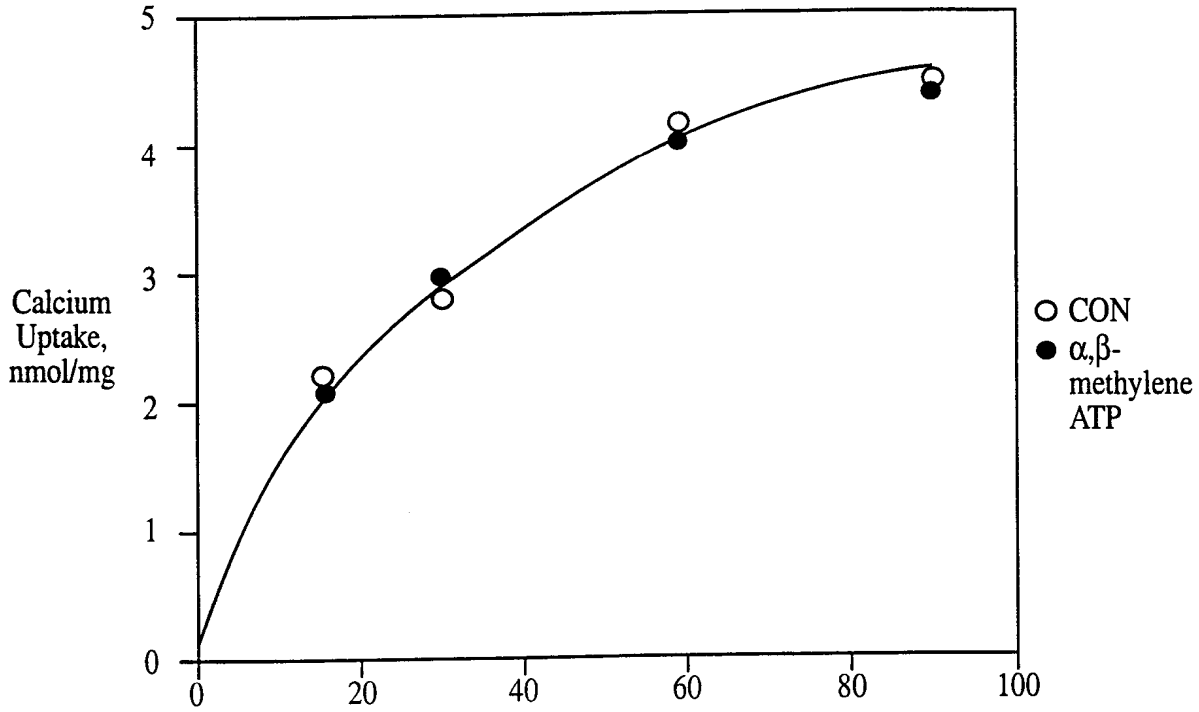
Calcium Uptake, nmol/mg
0
1
2
3
4
5
0
20
40
60
80
100
CON
α,β-methylene ATP

Fig. 7B

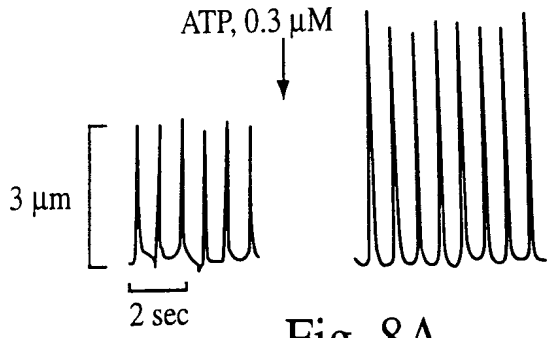

Fig. 8A

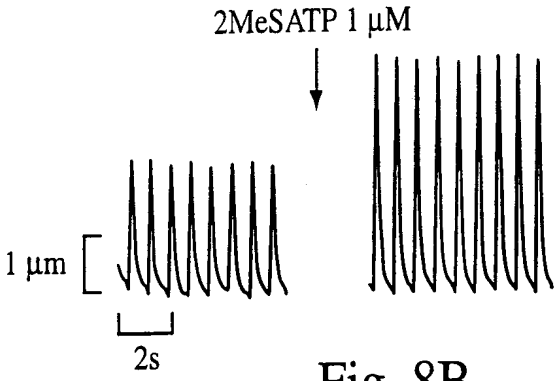

Fig. 8B

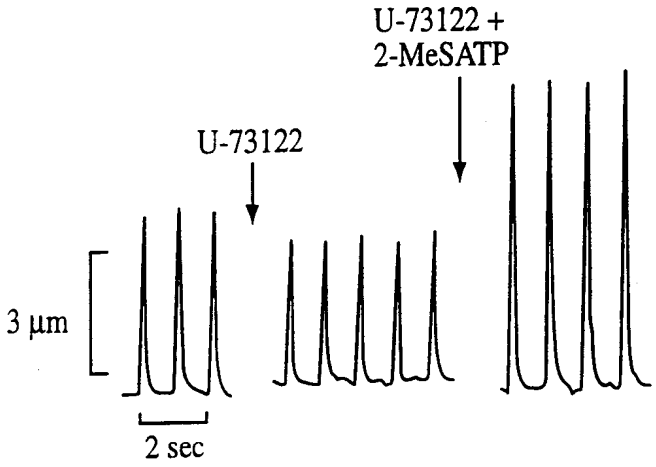

Fig. 9C

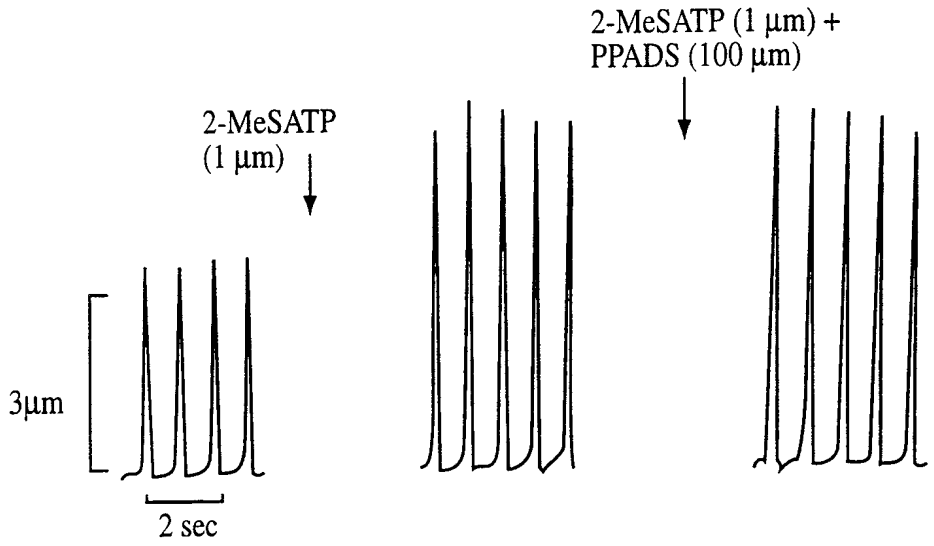
2-MeSATP (1 µm) +
PPADS (100 µm)
2-MeSATP
(1 µm)
3µm
2 sec

Fig. 9A

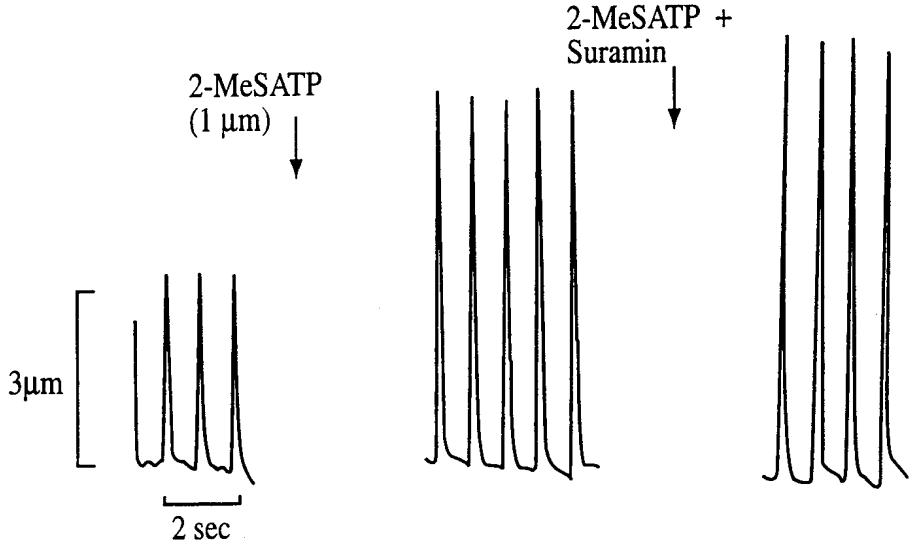
2-MeSATP +
Suramin
2-MeSATP
(1 µm)
3µm
2 sec

Fig. 9B

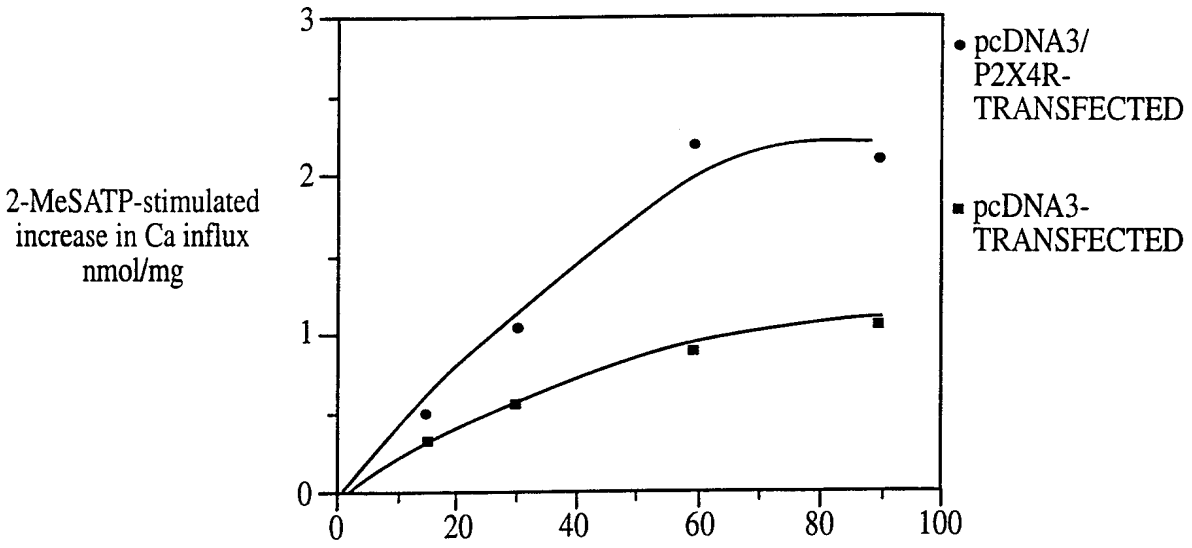
2-MeSATP-stimulated increase in Ca influx nmol/mg
3
2
1
0
0
20
40
60
80
100
pcDNA3/ P2X4R- TRANSFECTED
pcDNA3- TRANSFECTED

Fig. 10

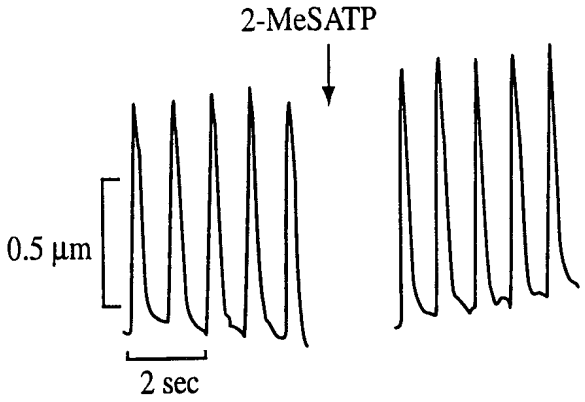

Fig. 11A

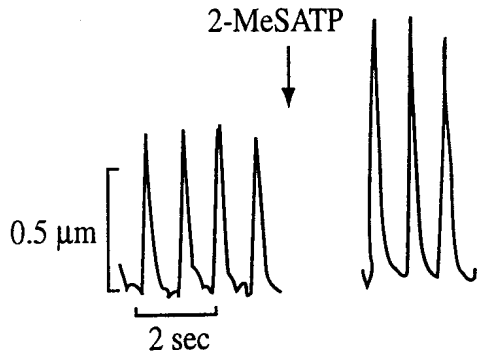

Fig. 11D

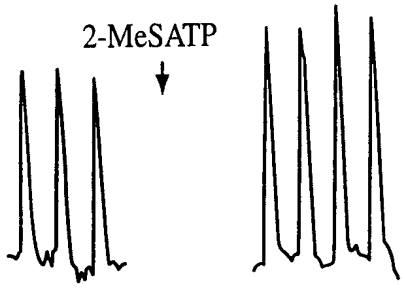

Fig. 11B

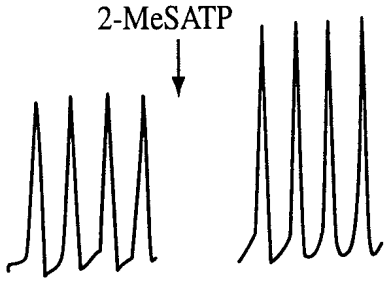

Fig. 11E

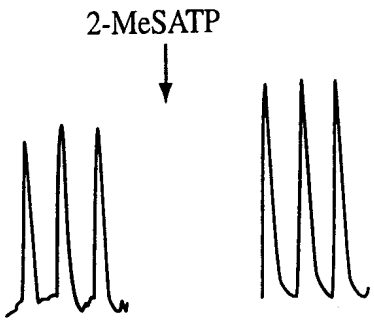

Fig. 11C

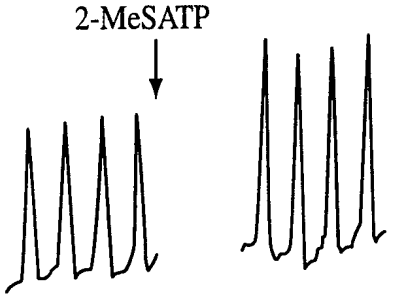

Fig. 11F

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23170

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/00, 15/12; A61K 39/00
US CL : 435/69.1, 320.1, 326; 530/350; 536/23.5; 514/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 320.1, 326; 530/350; 536/23.5; 514/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CAPLUS
search terms: myocyte, muscle, purinoreceptor, P2X, P2Y, cardiac, contractility

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MALAM-SOULEY, R. et al. Exogenous ATP induces a limited cell cycle progression of arterial smooth muscle cells. American Journal of Physiology. 1993, Vol. 264, No. 4 Part I pages C783-C788, see abstract.	1, 2, 4, 10-11, 34
Y	PACAUD, P. et al. ATP raises [Ca2+]i via different P2-receptor subtypes in freshly isolated and cultured aortic myocytes. American Journal of Physiology. 1995, Vol. 269, No. 1, Part 2, pages H30-H36, see abstract and page H35, last paragraph.	1, 2, 4, 10-11, 34

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 FEBRUARY 1999	Date of mailing of the international search report 02 MAR 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer D. Lawrence for ELIANE LAZAR-WESLEY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23170

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHANG, K. et al. Molecular cloning and functional analysis of a novel P2 nucleotide receptor. Journal of biological Chemistry. 03 November 1995, Vol. 270, No. 44, pages 26152-26158, especially page 26157, paragraph bridging col.1 and col.2.	1, 2, 4, 10-11, 34
Y	TOKUYAMA, Y. et al. Cloning of rat and mouse P2Y purinoreceptors. Biochemical and Biophysical Research Communications. 06 June 1995, Vol. 211, No. 1, pages 211-218, especially page 213.	1, 2, 4, 10-11, 34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23170

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-7, 10-11,33-36 as they relate to the first species, i.e. P2Y purinoreceptor.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23170

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-7, 10-11, and 33-36, drawn to a recombinant myocyte comprising at least one introduced nucleic acid encoding at least one P2 purinoreceptor, a method comprising assessing the contractile amplitude of a recombinant myocyte, and a kit. Eight species.

Group II, claims 8-9 and 12-13 , drawn to a method comprising assessing calcium uptake. Forty-eight species.

Group III, claims 14-32 , drawn to a method of enhancing cardiac contractility comprising administering to an animal a composition comprising purinoreceptors agonists and P2 purinoreceptor antagonists other than P2X4 and P2X6 antagonists, a pharmaceutical composition and a kit. Seven species.

Group IV, claim 37, drawn to a method of decreasing cardiac contractility comprising administering to an animal a purinoreceptor antagonist selected from a P2X4 and a P2X6 purinoreceptor antagonist. Two species.

The special technical feature of group I is the recombinant monocyte and the method of assessing contractility. The inventions of Groups I-IV lack unity of invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. The methods of Groups II-IV constitute different methods using different reagents and method steps and have special technical features different from the special technical features of Group I.

This application contains claims directed to more than one species of purinoreceptor used in the method of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species of purinoreceptors are as follows:
a)P2Y
b)P2X1
c)P2X2
d)P2X3
e)P2X4
f)P2X5
g)P2X6
h)P2X4/P2X6 heterodimer

The claims are deemed to correspond to the species listed above in the following manner:

a-g, claims 1-13, 33-36
a-h, claims 10-13

The following claims are generic: 1,2,4

Furthermore, claims 10-13 recite a second myocyte comprising at least one first purinoreceptor and at least one introduced nucleic acid encoding at least one second purinoreceptor.
These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:
a)P2Y
b)P2X1
c)P2X2
d)P2X3
e)P2X4
f)P2X5
g)P2X6
There appear to be forty-eight pairs of receptors.
The species listed above do not relate to a single inventive concept under PCT Rule 13.1, because under Rule PCT 13.2, the species lack the sanme or corresponding special technical features for the following reasons: they are unrelated in structure and activity.

Furthermore, this application contains claims directed to more than one species of purinoreceptor agonists and antagonists of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:
i)P2X4 purinoreceptor agonist
j)P2X6 purinoreceptor agonist
k)P2Y purinoreceptor antagonist
l)P2X1 purinoreceptor antagonist
m)P2X2 purinoreceptor antagonist
n)P2X3 purinoreceptor antagonist
o)P2X5 purinoreceptor antagonist
p)P2X4 purinoreceptor antagonist
q)P2X6 purinoreceptor antagonist

The claims are deemed to correspond to the species listed above in the following manner:

i-o, claims 14-32
p-q, claim 37

The following claims are generic: 14, 26

The species listed above do not relate to a single inventive concept under PCT Rule 13.1, because under Rule PCT 13.2, the species lack the same or corresponding special technical features for the following reasons: they are unrelated in structure and mode of action.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the reasons cited above.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the reasons cited above.